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(54) Title: RNA INTERFERENCE BY PALINDROMIC AND LABELLED RNA MOLECULES

(57) Abstract: The present invention relates to sequence and structural features of RNA molecules required to mediate target-specific nucleic acid modifications by RNA-interference (RNAi), such as target mRNA degradation and/or DNA methylation.

WO 2004/065600 A2

# RNA Interference by Palindromic and Labelled RNA Molecules

## Description

The present invention relates to sequence and structural features of double-stranded (ds) RNA molecules required to mediate target-specific nucleic acid modifications by RNA-interference (RNAi), such as target mRNA degradation and/or DNA methylation.

Double-stranded RNA (dsRNA) is an important signaling molecule in eukaryotic cells. It triggers an elegant defense system that evolved to eliminate unwelcome foreign RNA molecules such as cytoplasmic replicating RNA viruses and transcripts originating from transgenes, transposable elements or viruses randomly integrated into the host genome (Waterhouse et al., 2001; Grishok and Mello, 2002; Hannon, 2002; Mlotshwa et al., 2002; Plasterk, 2002; Tijsterman et al., 2002b; Voinnet, 2002; Zamore, 2002). During evolution, Animals and plants have also started to express genes that encode short dsRNAs, so called microRNAs, which control cellular processes and developmental events (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee et al., 2002b; Llave et al., 2002; Moss and Poethig, 2002; Pasquinelli and Ruvkun, 2002; Reinhart et al., 2002). Furthermore, dsRNA confers epigenetic information by regulating heterchromatin formation (Dernburg and Karpen, 2002; Hall et al., 2002; Jenuwein, 2002; Reinhart and Bartel, 2002; Volpe et al., 2002), and DNA methylation (Jones et al., 1999; Sijen and Kooter, 2000; Wassenegger, 2000; Aufsatz et al., 2002) as well as directed elimination of DNA sequences Tetrahymena thermophila (Mochizuki et al., 2002).

In animals, dsRNA acts predominantly through a posttranscriptional mechanism targeting mature mRNAs for degradation; this form of RNA silencing is commonly called RNA interference (RNAi) (Fire et al., 1998).

The mediators for sequence-specific target recognition are approximately 21-nucleotide (nt) small interfering RNAs (siRNAs), which are produced from long dsRNA by a processing reaction involving Dicer RNase III (Bernstein et al., 2001; Elbashir et al., 2001a; Zamore, 2001). The siRNA duplexes are subsequently unwound to form single-strand containing ribonucleoprotein complexes that guide cytoplasmic target mRNA degradation (RNA-induced silencing complex, RISC) (Hammond et al., 2000; Martinez et al., 2002a; Schwarz et al., 2002) or related complexes that guide sequence-specific DNA or chromatin changes. In organisms that carry RNA-dependent RNA polymerase (RdRP) genes, such as C. elegans, it is conceivable that siRNAs also prime dsRNA synthesis using the target mRNA as template, and that the target RNA is then inactivated by Dicer RNA cleavage rather than RISC (Sijen et al., 2001; Tijsterman et al., 2002a). In organisms lacking RdRP genes, e.g. D. melanogaster or mammals, replicative mechanisms for RNAi signals do not seem to exist (Chiu and Rana, 2002; Kisielow et al., 2002; Martinez et al., 2002a; Schwarz et al., 2002) and the efficiency of the silencing process is explained by the catalytic target mRNA cleavage by RISC (Hutvágner and Zamore, 2002).

In somatic mammalian cells, exogenously delivered dsRNAs of less than 30 base pairs activate a sequence-unspecific response, also called the interferon response (Lengyl, 1987). However, duplexes of 2l-nt siRNAs bypass this response and specifically silence genes expressed in cultured mammalian cells without apparent sequence-unspecific effects (Bitko and Barik, 2001; Caplen et al., 2001; Elbashir et al., 2001a; Harborth et al., 2001). The sense and antisense siRNA strands are paired such that a 2-nt 3′ overhang is formed (Elbashir et al., 2001a; Elbashir et al., 2002). In order to guide target RNA degradation, 5′ phosphorylation of the target-complementary siRNA strand is required (Nykänen et al., 2001; Schwarz et al., 2002). As long as the 5′-hydroxyl of an siRNA is not blocked by methylation or a 5′-phosphodiester linkage, a cellular kinase rapidly 5′-

phosphorylates the siRNA duplex (Chiu and Rana, 2002; Martinez et al., 2002a; Schwarz et al., 2002).

RISC contains single-stranded siRNA (Martinez et al., 2002a; Schwarz et al., 2002), and the siRNA duplex undergoes an ATP-dependent unwinding step prior to formation of RISC (Nykänen et al., 2001). Single-stranded 5'-phosphorylated antisense siRNAs are able to bypass this step and can enter RISC directly, although typically a higher concentration of single-stranded siRNA is required relative to duplex siRNA (Martinez et al., 2002a; Schwarz et al., 2002). The target mRNA is cleaved in the center of complementarity with the guide siRNA, whereby the 5' end of the guide siRNA, not the 3' end, directs the cleavage precisely 10 nt upstream from the complementary residue to the guide siRNA 5' end (Elbashir et al., 2001b). RISC contains protein members of the Argonaute family, Ago2 in D. melanogaster (Hammond et al., 2001) and eIF2C1 and/or eIF2C2 in human (Hutvágner and Zamore, 2002; Martinez et al., 2002a). Target RNA cleavage by RISC is ATP-independent (Nykänen et al., 2001; Hutvágner and Zamore, 2002). RISC has not yet been reconstituted from recombinant protein sources and the endonuclease activity remains to be identified; Dicer RNase III can be excluded because Dicer immuno-depleted extracts still reconstitute RISC on siRNAs (Martinez et al., 2002a).

siRNA duplexes are now widely used for silencing of mammalian genes in cultured cells (Harborth et al., 2001; Elbashir et al., 2002; McManus and Sharp, 2002; Paddison and Hannon, 2002). Most conveniently, chemically synthesized siRNA duplexes are used, although siRNAs can also be prepared enzymatically by in vitro transcription from short synthetic DNA templates (Donzé and Picard, 2002; Paddison et al., 2002; Yu et al., 2002) or by limited RNase III digestion from long dsRNA substrates (Calegari et al., 2002; Yang et al., 2002). Alternatively, hairpin RNAs with stem-lengths of 20 to 29 base pairs may be prepared that will later be processed inside the cell to siRNAs (McManus et al., 2002; Paddison et al., 2002; Yu

et al., 2002). Annealed duplex RNAs are generally transfected in cultured somatic cells using various classical or specifically developed liposome-based transfection reagents (Elbashir et al., 2002; Lassus et al., 2002; McManus and Sharp, 2002). Alternatively, siRNAs are delivered via electroporation into cells (Brummelkamp et al., 2002b) or organisms (Calegari et al., 2002; Hu et al., 2002). Silencing persists for several cell generations (i.e. several days) and simultaneous knockdown of more than one protein within the same cell population is possible (Elbashir et al., 2002; Holen et al., 2002).

An alternative strategy to RNA delivery is plasmid-based or viral vector-based expression of siRNAs or hairpin RNAs (for review see Tuschl, 2002). Short RNA sequences are typically transcribed using polymerase III promoters of U6 or H1 RNAs (Brummelkamp et al., 2002b; Lee et al., 2002a; McManus et al., 2002; Miyagishi and Taira, 2002; Paddison et al., 2002; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002). Alternatively, a strong polymerase II promoter, e.g. CMV, is suitable for hairpin expression (Xia et al., 2002; Zeng et al., 2002). Both, transient expression of plasmid-encoded siRNAs and direct delivery of synthetic siRNAs only produce transient knockdown of target genes. These approaches are also often limited to the use of cell lines that are readily transfected or electroporated. These disadvantages can be partly overcome by generating stable knockdown cells, which is currently only possible when non-essential genes are targeted (Brummelkamp et al., 2002b; Paddison et al., 2002). Development of an inducible siRNA expression constructs will overcome this last obstacle. Alternatives to plasmid-based expression systems are offered by inserting the expression cassettes into retroviral vectors (Barton and Medzhitov, 2002; Brummelkamp et al., 2002a; Devroe and Silver, 2002; Paddison and Hannon, 2002) and adenoviral vectors (Xia et al., 2002). It should however be noted that the retroviral LTRs, which are strong promoters elements themselves, may be mostly responsible for driving retroviral hairpin expression rather the promoter of the expression

cassette. The specificity of knockdown of a particular gene by a particular siRNA should be assessed by using two independent siRNA sequences cognate to the same target mRNA (Elbashir et al., 2002) or by rescuing the knockdown by ectopically expressing a RNAi-resistant cDNA of the targeted protein (Devroe and Silver, 2002; Lassus et al., 2002). This is either accomplished by introducing silent mutations in the complementing cDNA or by targeting the 3'-UTR of the endogene and preparing a rescue construct that only contains the open reading frame followed by a different 3'-UTR sequence, such as from SV40 large T antigen, frequently a part of mammalian cDNA expression vectors.

All siRNA or hairpin siRNA-based gene targeting experiments face the problem that they are directed against only a small segment of the target mRNA, and that it cannot be predicted if that selected targeting region is fully accessible to RISC. Target RNA self-structure and target RNA-binding proteins may influence accessibility. Variation of targeting efficiencies were documented for a small set of siRNAs but no satisfactory explanation was given to explain the variation (Harborth et al., 2001; Holen et al., 2002). Additionally, formation of RISC and product release after target RNA cleavage may be sequence-dependent. Furthermore, mistargeting of sequences closely related to the target has to be avoided and may compete away siRNAs needed for specific target recognition. Hairpin siRNAs introduce an additional level of complexity as the require Dicer processing to siRNAs.

Despite the discussion on variation of siRNA efficiency, effective siRNAs are identified at high frequency and the testing of siRNAs in animal models has not been hampered. siRNAs appear to enter highly vascularized tissues, especially liver, upon injection into the tail vein of mice (Lewis et al., 2002; McCaffrey et al., 2002). Tumor cells stably transduced with retroviral siRNA expression constructs directed against a mutant oncogenic form of RAS experienced loss of anchorage-independent growth and

tumorigenicity in a mouse model (Brummelkamp et al., 2002a). Recombinant adenoviruses expressing siRNAs under the control of a CMV promoter reduced target gene expression in mouse liver or brain, when injected into the tail vein or the brain striatal region of the animal, respectively (Xia et al., 2002). These early reports suggest new possibilities for gene-specific interventions of human disease (Borkhardt, 2002; Paddison and Hannon, 2002; Tuschl and Borkhardt, 2002).

To advance the reproducibility, effectiveness and specificity of the siRNA-based gene silencing technology, we have performed an extensive comparative analysis determining the positional variation of siRNA targeting efficiencies between the orthologous mouse and human lamin A/C gene of human HeLa and mouse SW3T3 cells. Furthermore, we have compared hairpin siRNAs to canonical siRNA duplexes and tested various chemical modifications known to enhance stability of RNA molecules. 2'-Fluoro-2'-deoxyribose substitutions as well as terminal thiophosphate linkages were tolerated in siRNAs and may provide, because of their nuclease stability, significant advantages for studies in animal models.

A first aspect of the present invention relates to a double-stranded RNA molecule wherein each RNA strand has a length of from 15-50 nucleotides, preferably from 18-30 nucleotides and more preferably from 19-25 nucleotides comprising an antisense strand which is at least substantially complementary to a target transcript and a sense strand which is at least substantially complementary to the antisense strand, wherein the RNA molecule has an at least partially palindromic sequence. The use of palindromic RNA molecules leads to a reduction of mistargeting and production costs.

The double-stranded RNA molecule or a metabolic processing product thereof is capable of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation. Preferably at least

one RNA strand has a 5' and/or 3' overhang. Preferably, one end of the double-strand has a 3' overhang from 1-5 nucleotides, more preferably from 1-3 nucleotides and most preferably 2 nucleotides. The other end may be blunt-ended or has up to 6 nucleotides 3' overhang.

The double-stranded RNA molecule comprises an antisense strand and a sense-strand. The antisense strand is at least substantially complementary to the target transcript. Preferably at least a portion of the antisense strand is completely complementary to the target transcript, wherein the complementary base pairs between target transcript and antisense strand preferably consist of the traditional Watson-Crick base pairs i.e. A/U, U/A, G/C and C/G, as well as G/U and U/G base pairs. The complementarity of the antisense strand with the target transcript is discussed in further detail below.

The double-stranded RNA molecule of the invention has an at least partially palindromic sequence, i.e. the sequence in one strand comprises at least a portion which is self-complementary. Preferably, the palindromic sequence has a length of from 10-30, more preferably from 10-24 nucleotides (referring to the whole palindromic sequence consisting of two complementary portions and optionally one central nucleotide). The degree of self-complementarity within the palindromic sequence is preferably at least 80%, more preferably at least 90% based on the number of nucleotides within the whole palindromic sequence. Most preferably, there is a complete self-complementarity within the palindromic sequence.

In one embodiment, the antisense strand of the double-stranded RNA molecule is completely complementary to the sense strand. In another embodiment, the antisense strand is not completely complementary to the sense strand, but sufficiently complementary that the formation of a double-stranded RNA molecule is stable under application conditions, particularly under physiological conditions at temperatures of 20-40°C.

Preferably, the formation of a double-stranded RNA is energetically favourable compared to the formation of a hairpin structure in a single RNA strand.

Preferred complementary base pairs between the sense and antisense strands include the traditional Watson-Crick base pairs i.e. A/U, U/A, G/C and C/G as well as non-Watson-Crick base pairs selected from G/U and U/6. Less preferred complementary base pairs are selected from U/U, C/U, U/C, A/A and G/G base pairs. The proportion of less preferred base pairs of the total complementary base pairs between the sense and antisense strands is preferably not more than 50%, more preferably not more than 40% and most preferably not more than 30%.

The degree of complementarity between the antisense strand and the sense strand is preferably at least 70%, more preferably at least 80% and most preferably at least 90% based on the total length of the double-stranded portion of the RNA molecule. For example, up to 8, preferably up to 6 and more preferably up to 4 mismatches over the whole length of the double strand may be tolerated.

Due to the palindromic nature of the RNA molecule both strands, i.e. the antisense strand and the sense strand may be substantially, i.e. at least 85%, preferably at least 90% and more preferably at least 95%, identical. In an especially preferred embodiment, both sense and antisense strands are completely identical. The high degree of identity between antisense and sense strand increases the target specificity and lowers the production costs.

A further aspect of the invention relates to a double-stranded RNA molecule wherein each RNA strand has a length of from 15-50 nucleotides, preferably from 18-30 nucleotides and more preferably 19-25 nucleotides comprising an antisense strand which is at least substantially

complementary to a target transcript and a sense strand which is at least substantially complementary to the antisense strand, and wherein the RNA carries at least one labelling group on the 5'- or the 3'-end of the sense strand or on the 5'-end of the antisense strand. The labelled RNA molecule is capable of mediating target-specific nucleic acid modifications as described above.

The labelling group may be coupled directly via a linker, wherein the linker preferably has a chain length of 4-20 atoms, e.g. carbon atoms and optionally including heteroatoms such as N, O or S atoms.

The labelling group is preferably a fluorophore, e.g. fluorescein or a derivative thereof, or another fluorophore. Labelled RNA molecules may be used for the sorting and isolation of cells which have been transfected with RNA molecules e.g. by FACS. Further, labelled RNA molecules may be used for diagnostic applications.

In this embodiment of the invention the RNA molecule may be a palindromic RNA molecule as described above. It should be noted, however, that also non-palindromic molecules are encompassed by this embodiment. Preferably, the RNA molecule of this embodiment comprises overhangs as described above.

Still a further embodiment of the present invention relates to a double-stranded RNA molecule wherein each RNA strand has a length from 15-50 nucleotides, preferably from 18-30 nucleotides and more preferably from 19-25 nucleotides comprising an antisense strand which is at least substantially complementary to a target transcript and a sense strand which is at least substantially complementary to the antisense strand and wherein the sense strand comprises a non-nucleotidic 5'- and/or 3'-modification which inhibits RNA interference.

The sense strand modifications may be combined with palindromic sequences and/or labelling groups. Examples from non-nucleotidic and/or modifications which inhibit RNA interference for the sequence represented by the sense strand are 3'-modifications on the sense strand, e.g. fluorophores which have been demonstrated to inhibit RNA interference.

In all embodiments of the invention the double-stranded RNA molecule as such or a metabolic procesing product thereof is capable of sequence-specific inhibition of target transcript expression. The inhibition of target transcript expression may occur in vitro, e.g. in eukaryotic, particularly mammalian cell cultures or cell extracts. On the other hand, the inhibition may also occur in vivo i.e. in eucaryotic, particularly mammalian organisms including human beings.

The individual strands of the double-stranded RNA molecule may have a 3´hydroxyl group. In some cases, however, it may be preferable to modify the 3' end of at least one strand to make it resistant against 3' to 5' exonucleases. Tolerated 3'-modifications are for example terminal 2'-deoxy nucleotides, 3' phosphate, 2',3'-cyclic phosphate, C3 (or C6, C7, C12) aminolinkers, thiol linkers, carboxyl linkers, non-nucleotidic spacers (C3, C6, C9, C12, abasic, triethylene glycol, hexaethylene glycol), biotin, fluorescein, etc. A further modification, by which the nuclease resistance of the RNA molecule may be increased, is by covalent coupling of inverted nucleotides, e.g. 2'-deoxyribonucleotides or ribonucleotides to the 3'-end of the RNA molecule. A preferred RNA strand structure comprises: 5'-siRNA-3'-O-P(O)(OH)-O-3'-N, wherein N is a nucleotide, e.g. a 2'-deoxyribonucleotide or ribonucleotide, typically an inverted thymidine residue, or an inverted oligonucleotide structure, e.g. containing up to 5 nucleotides.

The 5´-terminus of an individual strand may comprise an OH group, a phosphate group or an analogue thereof. Preferred 5' phosphate

modifications are 5'-monophosphate ($(HO)_2(O)P$-O-5'), 5'-diphosphate ($(HO)_2(O)P$-O-P(HO)(O)-O-5'), 5'-triphosphate ($(HO)_2(O)P$-O-(HO)(O)P-O-P(HO)(O)-O-5'), 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'), 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure (N-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'), 5'-monothiophosphate (phosphorothioate; $(HO)_2(S)P$-O-5'), 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ($(HO)_2(O)P$-S-5'); any additional combination of oxgen/sulfur replaced monophosphate, diphosphate and triphosphates (e.g. 5'-alpha-thiotriphosphate, 5'-gamma-thiotriphosphate, etc.), 5'-phosphoramidates ($(HO)_2(O)P$-NH-5', $(HO)(NH_2)(O)P$-O-5'), 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., e.g. RP(OH)(O)-O-5'-, $(OH)_2(O)P$-5'-$CH_2$-), 5'-alkyletherphosphonates (R=alkylether=methoxymethyl ($MeOCH_2$-), ethoxymethyl, etc., e.g. RP(OH)(O)-O-5'-).

The sequence of the RNA molecule of the present invention has to have a sufficient identity to a nucleic acid target molecule in order to mediate target-specific RNAi. Thus the antisense strand of the RNA molecule is substantially complementary to the target transcript.

The target RNA cleavage reaction guided by the RNA molecules of the present invention is highly sequence-specific. However, not all positions of the RNA molecule contribute equally to target recognition. Mismatches, particularly at the 3'-terminus of the antisense strand of the RNA molecule, more particularly at the residues 3' to the first 20 nt of the antisense strand of the RNA molecule are tolerated. Especially preferred are RNA molecules having at the 5'-terminus of the antisense strand at least 15 and preferably at least 20 nucleotides which are completely complementary to a predetermined target transcript or have at only mismatch and optionally up to 15 nucleotides at the 3'-terminus which may contain 1 or several, e.g. 2, 3 or more mismatches.

In order to enhance the stability of the RNA molecules, the 3´-ends may be stabilized against degradation, e.g. they may be selected such that they consist of purine nucleotides, particularly adenosine or guanosine nucleotides. Alternatively or additionally, nucleotides may be substituted by modified nucleotide analogues, including backbone modifications of ribose and/or phosphate residues.

In an especially preferred embodiment of the present invention the RNA molecule may contain at least one modified nucleotide analogue. The nucleotide analogues may be located at positions where the target-specific activity, e.g. the RNAi mediating activity is not substantially affected, e.g. preferably in a region at the 5'-end and/or the 3'-end of the RNA molecule.

Preferred nucleotide analogues are selected from sugar- or backbone-modified ribonucleotides. It should be noted, however, that also nucleobase-modified ribonucleotides, i.e. ribonucleotides, containing a non-naturally occurring nucleobase instead of a naturally occurring nucleobase such as uridines or cytidines modified at the 5-position, e.g. 5-(2-amino)propyl uridine, 5-bromo uridine; 5-methyl-cytidine; adenosines and guanosines modified at the 8-position, e.g. 8-bromo guanosine; deaza nucleotides, e.g. 7-deaza-adenosine; O- and N-alkylated nucleotides, e.g. N6-methyl adenosine are suitable. In preferred sugar-modified ribonucleotides the 2´ OH-group is replaced by a group selected from H, OR, R, halo, SH, SR, $NH_2$, NHR, $NR_2$ or CN, wherein R is $C_1$-$C_6$ alkyl, alkenyl, alkynyl or methoxyethoxy, and halo is F, Cl, Br or I, particularly F. In preferred backbone-modified ribonucleotides the phosphoester group connecting to adjacent ribonucleotides is replaced by a modified group, e.g. a phosphorothioate, phosphorodithioate, N3'-O5'- and/or N5'-O3' phosphoramidate group. It should be noted that the above modifications may be combined. For example, complementary or non-complementary nucleotides at the 3'-terminus, particularly after at least 15, more

particularly after at least 20 5'-terminal nucleotides may be modified without significant loss of activity.

Still a further embodiment of the invention relates to a double-stranded RNA molecule wherein each RNA strand has a length from 15-50, preferably from 18-30 nucleotides and more preferably from 19-25 nucleotides comprising an antisense strand which is at least substantially complementary to a target transcript and a sense strand which is at least substantially complementary to the antisense strand and wherein at least one strand, e.g. the antisense strand comprises a 2'-OH modified nucleotide, preferably a pyrimidine nucleotide e.g. a C and/or U nucleotide and a modified phosphoester group. Preferably both strands are modified. An individual strand of the double-stranded RNA molecule may contain preferably up to 20 and more preferably up to 15 2'OH modified nucleotides. For example, all pyrimidine nucleotides may contain 2'OH modifications, such as halo, R, OR, SH, SR, $Nh_2$, NHR, $NR_2$ or CN as described above. A preferred 2'OH modification is the 2' fluoro group. A further preferred 2' OH modification is the 2'H group (deoxy-ribonucleotide), which can be present in preferably up to 4 nucleotides of an RNA strand. An individual strand of the double-stranded RNA molecule may contain preferably up to 10, more preferably up to 5 modified phosphoester groups. For example, 1, 2, 3, 4 or 5 3' terminal phosphoester groups may be modified. A preferred phosphoester modification is the phosphorothiate (thiophosphate) group. The modified RNA molecules may contain further features as described above for other embodiments.

The RNA molecules of the invention may be prepared by chemical synthesis. Methods of synthesizing RNA molecules are known in the art.

The RNAs can also be prepared by enzymatic transcription from synthetic DNA templates or from DNA plasmids isolated from recombinant bacteria

and optionally subsequent 5´-terminal modification. Typically, phage RNA polymerases are used such as T7, T3 or SP6 RNA polymerase.

A further aspect of the present invention relates to a method of mediating RNA interference in a cell or an organism comprising the steps:

(a) contacting the cell or organism with the RNA molecule of the invention under conditions wherein target-specific nucleic acid modifications may occur and

(b) mediating a target-specific nucleic acid modification effected by the RNA towards a target nucleic acid having a sequence portion substantially complementary to the RNA.

Preferably the contacting step (a) comprises introducing the RNA molecule into a target cell, e.g. an isolated target cell, e.g. in cell culture, a unicellular microorganism or a target cell or a plurality of target cells within a multicellular organism. More preferably, the introducing step comprises a carrier-mediated delivery, e.g. by liposomal carriers and/or by injection. Further suitable delivery systems include Oligofectamine (Invitrogen) and Transit-TKO siRNA Transfection reagent (Mirus).

The method of the invention may be used for determining the function of a gene in a cell or an organism or even for modulating the function of a gene in a cell or an organism, being capable of mediating RNA interference. The cell is preferably a eukaryotic cell or a cell line, e.g. a plant cell or an animal cell, such as a mammalian cell, e.g. an embryonic cell, a pluripotent stem cell, a tumor cell, e.g. a teratocarcinoma cell or a virus-infected cell. The organism is preferably a eukaryotic organism, e.g. a plant or an animal, such as a mammal, particularly a human.

The target gene to which the RNA molecule of the invention is directed may be associated with a pathological condition. For example, the gene may be a pathogen-associated gene, e.g. a viral gene, a tumor-associated gene or an autoimmune disease-associated gene. The target gene may also be a heterologous gene expressed in a recombinant cell or a genetically altered organism. By determinating or modulating, particularly, inhibiting the function of such a gene valuable information and therapeutic benefits in the agricultural field or in the medicine or veterinary medicine field may be obtained.

The present invention also allows a highly specific targeting of transcripts in a cell or in an organism, e.g. the targeting of individual transcript isoforms or transcript polymorphisms.

The RNA is usually administered as a pharmaceutical composition. The administration may be carried out by known methods, wherein a nucleic acid is introduced into a desired target cell in vitro or in vivo. Commonly used gene transfer techniques include calcium phosphate, DEAE-dextran, electroporation and microinjection and viral methods (Graham, F.L. and van der Eb, A.J. (1973) Virol. 52, 456; McCutchan, J.H. and Pagano, J.S. (1968), J. Natl. Cancer Inst. 41, 351; Chu, G. et al (1987), Nucl. Acids Res. 15, 1311; Fraley, R. et al. (1980), J. Biol. Chem. 255, 10431; Capecchi, M.R. (1980), Cell 22, 479). A recent addition to this arsenal of techniques for the introduction of nucleic acids into cells is the use of cationic liposomes (Felgner, P.L. et al. (1987), Proc. Natl. Acad. Sci USA 84, 7413). Commercially available cationic lipid formulations are e.g. Tfx 50 (Promega) or Lipofectamin2000 (Life Technologies). A further preferred method for the introduction of RNA into a target organism, particularly into a mouse, is the high-pressure tail vein injection (Lewis, D.L. et al. (2002), Nat.Genet.29, 29; McCaffrey, A.P. et al. (2002), Nature 418, 38-39). Herein, a buffered solution comprising the double-stranded RNA (e.g. about 2 ml) is injected into the tail vein of the mouse within 10 s.

Thus, the invention also relates to a pharmaceutical composition containing as an active agent at least one RNA molecule as described above and pharmaceutically acceptable carriers, diluents and/or adjuvants. The composition may be used for diagnostic and for therapeutic applications in human medicine or in veterinary medicine.

For diagnostic or therapeutic applications, the composition may be in form of a solution, e.g. an injectable solution, a cream, ointment, tablet, suspension or the like. The composition may be administered in any suitable way, e.g. by injection, by oral, topical, nasal, rectal application etc. The carrier may be any suitable pharmaceutical carrier. Preferably, a carrier is used, which is capable of increasing the efficacy of the RNA molecules to enter the target-cells. Suitable examples of such carriers are liposomes, particularly cationic liposomes. A further preferred administration method is injection.

A further preferred application of the RNAi method is a functional analysis of eukaryotic cells, or eukaryotic non-human organisms, preferably mammalian cells or organisms and most preferably human cells, e.g. cell lines such as HeLa or 293 or rodents, e.g. rats and mice. By transfection with suitable RNA molecules which are homologous to a predetermined target gene or DNA molecules encoding a suitable RNA molecule a specific knockout phenotype can be obtained in a target cell, e.g. in cell culture or in a target organism. The presence of short double-stranded RNA molecules does not result in an interferon response from the host cell or host organism.

The RNA molecule may be administered associated with biodegradable polymers, e.g. polypeptides, poly(d,l-lactic-co-glycolic acid) (PLGA), polylysine or polylysine conjugates, e.g. polylysine-graft-imidazole acetic acid, or poly(beta-amino ester) or microparticles, such as microspheres, nanoparticles or nanospheres. The RNA molecule may be covalently

coupled to the polymer or microparticle, wherein the covalent coupling particularly is effected via the 3´-terminus of a strand of the RNA molecule.

Furthermore, the invention relates to a method for the monitoring, prevention or treatment of a disease associated with overexpression of at least one target gene comprising administering a subject in need thereof an RNA molecule as described above.

Still, a further subject matter of the invention is a eukaryotic cell or a eukaryotic non-human organism exhibiting a target gene-specific knockout phenotype comprising an at least partially deficient expression of at least one endogeneous target gene wherein said cell or organism is transfected with at least one RNA molecule capable of inhibiting the expression of at least one endogeneous target gene or a DNA encoding RNA. It should be noted that the present invention allows the simultaneous delivery of several antisense RNAs of different sequences, which are either cognate to a different or the same target gene.

Gene-specific knockout phenotypes of cells or non-human organisms, particularly of human cells or non-human mammals may be used in analytic procedures, e.g. in the functional and/or phenotypical analysis of complex physiological processes such as analysis of gene expression profiles and/or proteomes. For example, one may prepare the knock-out phenotypes of human genes in cultured cells which are assumed to be regulators of alternative splicing processes. Among these genes are particularly the members of the SR splicing factor family, e.g. ASF/SF2, SC35, SRp20, SRp40 or SRp55. Further, the effect of SR proteins on the mRNA profiles of predetermined alternatively spliced genes such as CD44 may be analysed. Preferably the analysis is carried out by high-throughput methods using oligonucleotide based chips.

Using RNAi based knockout technologies, the expression of an endogeneous target gene may be inhibited in a target cell or a target organism. The endogeneous gene may be complemented by an exogeneous target nucleic acid coding for the target protein or a variant or mutated form of the target protein, e.g. a gene or a cDNA, which may optionally be fused to a further nucleic acid sequence encoding a detectable peptide or polypeptide, e.g. an affinity tag, particularly a multiple affinity tag. Variants or mutated forms of the target gene differ from the endogeneous target gene in that they encode a gene product which differs from the endogeneous gene product on the amino acid level by substitutions, insertions and/or deletions of single or multiple amino acids. The variants or mutated forms may have the same biological activity as the endogeneous target gene. On the other hand, the variant or mutated target gene may also have a biological activity, which differs from the biological activity of the endogeneous target gene, e.g. a partially deleted activity, a completely deleted activity, an enhanced activity etc.

The complementation may be accomplished by coexpressing the polypeptide encoded by the exogeneous nucleic acid, e.g. a fusion protein comprising the target protein and the affinity tag and the RNA molecule for knocking out the endogeneous gene in the target cell. This coexpression may be accomplished by using a suitable expression vector expressing both the polypeptide encoded by the exogeneous nucleic acid, e.g. the tag-modified target protein and the RNA molecule or alternatively by using a combination of expression vectors. Proteins and protein complexes which are synthesized de novo in the target cell will contain the exogeneous gene product, e.g. the modified fusion protein. In order to avoid suppression of the exogeneous gene product expression by the RNAi molecule, the nucleotide sequence encoding the exogeneous nucleic acid may be altered on the DNA level (with or without causing mutations on the amino acid level) in the part of the sequence which is homologous to the RNA molecule. Alternatively, the endogeneous target gene may be

complemented by corresponding nucleotide sequences from other species, e.g. from mouse.

Preferred applications for the cell or organism of the invention is the analysis of gene expression profiles and/or proteomes. In an especially preferred embodiment an analysis of a variant or mutant form of one or several target proteins is carried out, wherein said variant or mutant forms are reintroduced into the cell or organism by an exogeneous target nucleic acid as described above. The combination of knockout of an endogeneous gene and rescue by using mutated, e.g. partially deleted exogeneous target has advantages compared to the use of a knockout cell. Further, this method is particularly suitable for identifying functional domains of the target protein. In a further preferred embodiment a comparison, e.g. of gene expression profiles and/or proteomes and/or phenotypic characteristics of at least two cells or organisms is carried out. These organisms are selected from:

(i) a control cell or control organism without target gene inhibition,

(ii) a cell or organism with target gene inhibition and

(iii) a cell or organism with target gene inhibition plus target gene complementation by an exogeneous target nucleic acid.

The method and cell of the invention may also be used in a procedure for identifying and/or characterizing pharmacological agents, e.g. identifying new pharmacological agents from a collection of test substances and/or characterizing mechanisms of action and/or side effects of known pharmacological agents.

Thus, the present invention also relates to a system for identifying and/or characterizing pharmacological agents acting on at least one target protein comprising:

(a) a eukaryotic cell or a eukaryotic non-human organism capable of expressing at least one endogeneous target gene coding for said target protein,

(b) at least one RNA molecule capable of inhibiting the expression of said at least one endogeneous target gene by RNAi and

(c) a test substance or a collection of test substances wherein pharmacological properties of said test substance or said collection are to be identified and/or characterized.

Further, the system as described above preferably comprises:

(d) at least one exogeneous target nucleic acid coding for the target protein or a variant or mutated form of the target protein wherein said exogeneous target nucleic acid differs from the endogeneous target gene on the nucleic acid level such that the expression of the exogeneous target nucleic acid is substantially less inhibited by the RNA molecule than the expression of the endogeneous target gene.

Furthermore, the RNA knockout complementation method may be used for preparative purposes, e.g. for the affinity purification of proteins or protein complexes from eukaryotic cells, particularly mammalian cells and more particularly human cells. In this embodiment of the invention, the exogeneous target nucleic acid preferably codes for a target protein which is fused to an affinity tag.

The preparative method may be employed for the purification of high molecular weight protein complexes which preferably have a mass of $\geq$ 150 kD and more preferably of $\geq$ 500 kD and which optionally may contain nucleic acids such as RNA. Specific examples are the heterotrimeric protein complex consisting of the 20 kD, 60 kD and 90 kD proteins of the U4/U6 snRNP particle, the splicing factor SF3b from the 17S U2 snRNP consisting of 5 proteins having molecular weights of 14, 49, 120, 145 and 155 kD and the 25S U4/U6/U5 tri-snRNP particle containing the U4, U5

and U6 snRNA molecules and about 30 proteins, which has a molecular weight of about 1.7 MD.

Further, the present invention is explained in more detail in the following Figures and Examples.

**Figures**

Figure 1 shows several examples of palindromic double-stranded RNA molecules of the invention.

A and E The double-stranded siRNA has a 19 base pair double-standed palindromic portion consisting of a central nucleotide and 9 completely self-complementary nucleotides on each side. The anti-sense (AS) strand is completely complementary to the target mRNA and to the sense strand. The antisense strand and sense strand are identical.

B, C and D The antisense strands are completely complementary to the target mRNA. The sense strand and antisense strand are palindromic, but not completely complementary. The antisense strand and sense strand are identical.

FIG. 2. Alignment of human and mouse lamin A cDNA sequences. The open reading frame and the complete 3'-UTRs of mouse (upper line) and human (lower line) lamin A are shown. The GenBank accession numbers refer to the exonic sequences of human and mouse lamin A. Differences between human and mouse sequences are shadowed in yellow. The position at which lamin A and lamin C diverge is indicated by arrowheads and a capital C; the mouse lamin C terminus is completely different from the lamin A terminus, whereas the human lamin C sequence is characterized by a 121 nt insert (containing a stop codon) followed again

by the lamin A 3' sequence. The 23-nt segments used to design the siRNAs are indicated and designated according to the position relative to the start codon. Green bars indicate siRNAs cognate to both mouse and human lamin A/C sequence, a light green bar indicates a mouse-specific siRNA and blue bars indicate human lamin A/C specific siRNAs. The orange bar indicates the region covered by the single-nucleotide spaced walk. The position of the stop codon is highlighted in gray. Single-nucleotide polymorphisms for human lamin A are indicated by asterisks and were retrieved from the NCBI SNP database (http://www.ncbi.nlm.gov/SNP/snp_ref.cgi?locusId=4000).

Six to eight independently picked clones of SW3T3 and HeLa lamin A cDNA were sequenced between the position 57 to 2000 for SW3T3 and 57 to 2336 for HeLa. Nucleotide differences between the shown database entry are indicated by indicating the altered nucleotide above (for SW3T3) or below (for HeLa) the aligned database sequences.

FIG. 3. Common siRNA duplexes targeting different regions of human and mouse lamin A/C.

Human HeLa SS6 and mouse SW3T3 cells were transfected with specific and control siRNAs in parallel and harvested after 46 h using TransIT-TKO. The siRNA duplexes are named according to their targeting sites shown in Fig. 2; GL2, control luciferase siRNA duplex (A) Western blot using lamin A/C specific antibody and vimentin specific antibody. The blot was probed with lamin A/C and vimentin antibody in parallel, except for siRNA 2148 (indicated by an asterisk). Here, the blot was stripped and then reprobed for vimentin using different exposure times for luminescence quantification. (B) Quantification of lamin A/C knockdown after Western blot analysis. h608G10C refers to a G/C transition mutation, where position 10 (counting from the 5' end of the antisense siRNA) was changed from G to C and the sense siRNA compensatory change was performed to restore base-pairing. The Western blotting signal was quantified using a Lumi-Imager.

Differences in gel loading were normalized to non-targeted vimentin protein. Values are averages from lamin A and lamin C expression. The values for human HeLa SS6 cells are indicated in light gray and for mouse SW3T3 cells in dark gray. The nonspecific control GL2 luciferase siRNA duplex was used for normalization (black and white bars). Error bars represent standard deviations.

FIG. 4. Efficiencies of different siRNA duplexes on silencing of lamin A expression.

(A) long and (B) short distance walk. HeLa SS6 cells were transfected with specific and control siRNAs in parallel and harvested after 64 h and 44 h, respectively. Data are averages of at least three independent experiments. See also Fig. 3 for legend.

FIG. 5. Comparison of single- and double-stranded siRNA gene silencing. Lamin A expression after transfection of 5'-phosphorylated antisense, pas; 5'-phosphorylated duplex siRNAs, pds; and non-phosphorylated duplex siRNA, ds. Quantification of lamin A knockdown was carried out as described in Fig. 3. HeLa SS6 cells were transfected with specific and GL2 control siRNAs and harvested after 46h.

FIG. 6. Silencing effects of fluorophore-labeled lamin A/C siRNA duplexes. h608 siRNA duplexes were fluorescein- or Alexa488 -labeled at the sense or the antisense strand at the 5' or the 3' termini as indicated. The labeled siRNA duplexes were transfected into HeLa SS6 cells and silencing and fluorophore localization was analyzed after 38 h by immunfluorescence microscopy. A GL2 luciferase siRNA duplex was labeled at the 5' end of the sense strand and used as control. Hoechst staining was used to visualize nuclear chromatin. The white arrow indicates a transfected cell that does however not show silencing.

FIG. 7. Subcellular localization of Alexa488-labeled siRNA duplex after Oligofectamine transfection. h608 siRNA duplex 3' labeled at the anti-sense strand was transiently transfected into HeLa SS6 and localization analyzed after 20 h by fluorescence microscopy (left). Phase contrast microscopy (middle) shows the position of the cell nucleus. The overlay (right) shows, that labeled siRNA duplexes are predominantly localized in the cytoplasm outside of the nucleus.

FIG. 8. Lamin A/C silencing using thiophosphate-modified siRNAs. (A) Mouse siRNA sequence and modification. siRNAs were either modified at every second position, ps(even#); the 3' end, ps(19-21); or at the 5' end ps(2). Sense and antisense strand are indicated by s and as, respectively. (B) Reduction of lamin A/C in mouse SW3T3 cells 68 h past transfection. The blot was probed with lamin A/C and vimentin antibody in parallel. Data processing was as described in Fig. 3. Values are averages from three independent experiments. (C) Human siRNA sequence and modification. For symbols, see above (D) Single-stranded siRNAs (200 nM) or double-stranded siRNA duplexes (100 nM) with and without phosphorothioate modifications were transfected into HeLa SS6 cells. Quantification of lamin A/C knockdown was carried out by Western blot analysis 46 h after transfection.

FIG. 9. Lamin A/C silencing using 2'-fluoro-2'-deoxyribose-modified siRNAs. (A) Human siRNA sequence and modification. 5'-phosphorylated, 5'-p; 3'-phosphorothioate modification, ps; 2'-fluoro-2'-deoxyribose modification, f. (B) siRNAs were transiently transfected into HeLa SS6 and lamin A/C expression analyzed after 46 h as described in Fig. 3. Each experiment was normalized to a GL2 siRNA duplexes that contained the same specific type of modification(s) present in the lamin A/C siRNA duplex.

FIG. 10. Silencing of lamin A expression induced by various types of hairpin RNAs. (A) Hairpin RNA sequences. R or L indicate the position of the loop sequence, the number indicates the length of duplex formation. Joining of the top sense strand to the bottom antisense strand is indicated by two dashes; the bottom strand corresponds to the antisense guide siRNA. (B) siRNAs and hairpin RNAs were transiently transfected into HeLa SS6 and lamin A/C expression analyzed after 44 h as described in Fig. 3. Data are averages of two or three independent experiments. (C) Structure of non-nucleotidic linkers replacing the UUCG tetranucleotide loop. A, UUCG tetraloop; B,etc. (D) Real-time RT/PCR analysis of lamin A/C mRNA reduction. The duplexes were transfected in triplicate into A549 cells. Two negative transfection controls were used as well as p53 for a positive control. shRNA duplexes, two negative controls, and one positive control were transfected at a final concentration of 100 nM complexes with 2 μg/mL of cationic lipids in growth media containing serum for 24 hours.

## Examples

### 1. MATERIALS AND METHODS

#### 1.1 Cell culture

Human Hela SS6, human A549 (lung carcinoma line) and mouse SW3T3 cells were grown at 37 °C in Dulbecco's modified Eagle medium supplemented with 10% FCS, penicillin and streptomycin. Cells were regularly passaged to maintain exponential growth. In the case of HeLa SS6 cells, a passage number of 30 was not exceeded because at higher passage numbers siRNA transfection efficiencies decreased. This phenomenon was not observed for SW3T3 cells. 24 hours before transfection, 50,000 cells were seeded per well of a 24-well plate in 532 µl of medium (Elbashir et al., 2002). 24 h later, when a confluency of approximately 40% was reached, transfection was performed using Oligofectamine (Invitrogen) or TransIT-TKO (Mirus) and a final concentration of 100 nM siRNA duplex or 200 nM single-stranded siRNA with respect to final tissue culture volume in the well (Elbashir et al., 2002). For Western blotting, transfected cells were harvested 2 to 3 days posttransfection by trypsination and centrifugation. Cells for immunofluorescence analysis were grown on coverslips and methanol-fixed (Elbashir et al., 2002). For RT/PCR analysis of hairpin RNA-mediated silencing, 20,000 A549 cells were seeded per well in 48-well plates the day before transfection. On the day of transfection A549 cells were approximately 70% confluent. The hairpin RNAs and control siRNA duplexes were transfected by combining 0.025 nmol of duplex RNA and 0.5 µl Oligofectamine per well of the 48-well plate according to the manufacturers protocol. After addition of the RNA/oligofectamine complex to the cells the final volume per well was 250 µl and the siRNA concentration was 100 nM. Cells were then incubated for another 24 h.

1.2 Determination of cell viability

Cell growth rates and viability were determined by using the CASY Model TT cell counter and analysis system (Schärfe System, Reutlingen, Germany). First, the tissue culture supernatant is removed temporarily and 200 µl trypsin-EDTA solution (Invitrogen) is added to one well of a 24-well plate in order to detached the cells. Excess trypsin is quenched by the addition of 350 µl from the removed tissue culture supernatant. 100 µl of the cell suspension was then transferred to a CASYtube containing 10 ml CASYton solution (Schärfe System) and followed by cell counting.

1.3 siRNA synthesis and purification

Lamin A/C siRNA targeting regions were selected from 23-nt target mRNA segments of the type AA(N21) or NA(N21) (N, any ribonucleotide) (Elbashir et al., 2002). The 2-nt 3'-overhangs were composed of dTdT or riboNdT as described in the text. 21-nt siRNAs and hairpin RNA oligonucleotides were synthesized using 5'-silyl, 2'-ACE phosphoramidites (Dharmacon) on modified ABI 394 synthesizers (Foster City, CA, USA) (Scaringe, 2001b, a). Thiophosphate linkages were introduced during the oxidation step using 0.05 M 3H-1,2-Benzodithiole-3-one-1,1-dioxide in acetonitrile (Beaucage reagent) (Glen Research, VI, USA). 2'-Fluoro-modified siRNAs and 2'-fluoro/thiophosphate-modified siRNAs were purchased from Dharmacon.

After RNA synthesis on a 0.2 µmole-scale column, the phosphate methyl group was removed by flushing the column with 2 ml of 0.2 M 2-carbamyol-2-cyanoethylene-1,1-dithiolate trihydrate in DMF/water (98:2 v/v) for 20 min at room temperature. The reagent was removed and the column rinsed with 10 ml water followed by 10 ml acetonitrile. The RNA was cleaved and eluted from the solid support by flushing with 2 ml of 40% aqueous methylamine over 2 min, collected in a screw-cap vial and incubated for 10 min at 55 °C. Subsequently, the 2' ACE protected oligoribonucleotide was dried down in an Eppendorf concentrator and redissolved in sterile 2'-deprotection buffer (400 µl of 100 mM acetate-

TEMED, pH 3.8, for a 0.2 μmole scale synthesis). For hairpin RNAs, the deprotection solution was incubated at 90 °C for 3 min in a dry heat block, subsequently removed from the block and allowed to cool to ambient temperature for 30 minutes. Standard 21-nt siRNAs were deprotected for 30 minutes at 60 °C. The RNAs were precipitated from the acetate-TEMED solution by adding 24 μl 5 M NaCl and 1.2 ml of absolute ethanol. Hairpin RNAs were purified by native gel electrophoresis, for which the pellet was dissolved in native gel loading buffer composed of 50.0% glycerol and 50.0% 1X TBE and separated for 3 hours at 150V at room temperature on a native 10% 29:1 acrylamide:bisacrylamide (ICN, Costa Mesa, CA, USA) gel (17 x 14 cm, 4 mm spacer) in 1x TBE running buffer. The hairpin RNA was visualized by UV shadowing and excised from the gel. The gel slice was then crushed and soaked in 10 ml of 0.3 M NaOAc overnight at its natural pH of 8.15. The hairpin RNA was then desalted by passing it over a C-18 reverse phase Sep-Pak cartridges (Waters, Milford, MA, USA) as described (Tuschl et al., 1993). The desalted pellet was dissolved in water and adjusted to a final concentration of 20 μM in 100 mM KOAc, 30 mM HEPES, 2 mM MgOAc (pH 7.4). Standard 21-nt RNAs were not gel purified and the pellet was dissolved in water after 2' ACE deprotection.

Fluorescent chromophores were conjugated to the 3'-end of siRNAs post-synthetically by reacting the aliphatic 3' 3-carbon-aminolinker with the succinimidyl esters of 6-(fluorescein-5-(and-6)-carboxamido) hexanoic acid (F-2181, Molecular Probes, OR, USA) or Alexa Fluor® 488 carboxylic acid (A20000, Molecular Probes). 5'-Fluorescein was introduced by coupling as a phosphoramidite (Cat-Nr. 10-5901, Glen Research, VI, USA). The predicted/calculated masses per charge [M+H]+ were 7211.3/7214.3 for the sense 5'-fluorescein conjugate, 7159.2/7161.3 for the antisense 5'-fluorescein conjugate, 7284.3/7285.1 for the sense 3'-fluorescein conjugate, 7232.2/7231.5 for the antisense 3'-fluorescein conjugate, and 7293.0/7294.2 [M+H+NH4]+ for the antisense 3'-Alexa488 conjugate.

The unpurified 2'-ACE RNA oligos were analyzed for quality via anion exchange HPLC analysis and were 85-90% full length. Quality control of unmodified siRNAs and hairpins was carried out by running 0.5 A260 units (16 μg) of RNA over a 20% 8 M urea denaturing polyacrylamide gel. The RNA bands were detected by UV shadowing at 254 nm, and only RNAs, for which a single intense full-length band is detected were used. Annealing of sense and antisense siRNAs was performed as described, followed by quality control on native agarose gels (Elbashir et al., 2002). The presence of thiophosphate linkages was confirmed by MALDI-TOF mass spectrometry using positive mode. The matrix is prepared from 1 μl of approximately 300 μM 21-nt RNA sample, 0.5 μl of 50 μM 19-nt standard, 0.5 μl 50 μM 24-nt standard, 7 μl 3-hydroxy-picolinic acid (0.5 mg/ml), 2 μl diammonium citrate (0.5 mg/ml) and a few grains of ammonium ion loaded Dowex 50 WX8 for exchanging sodium ions. 2 μl of the matrix are spotted on the MALDI-TOF sample carrier.

1.4 Western blotting

The HeLa cell pellets obtained from one well of a 24-well plate were resuspended in 800 μL of 10 mM Tris-HCl (pH 7.6)/140 mM NaCl/5 mM EDTA/ 25 μM β-mercaptoethanol/1% Triton X-100 and incubated for 1 min at room temperature. Cytoskeletal proteins, including lamin A/C and vimentin, were collected by centrifugation while soluble proteins remained in the supernatant. The cytoskeletal pellet was dissolved in SDS sample buffer (Bio-Rad, No. 161-073; 15 μl per well of a 24 well plate, corresponding to approx. 500,000 cells). Cell pellets of trypsinized SW3T3 cells were only washed with cold phosphate-buffered saline (PBS) and directly solubilized in SDS sample buffer. For HeLa and SW3T3 cells, SDS sample buffer-solutions were incubated for 3 min in boiling water and sonicated for 5 min in an ultrasonication bath at room temperature prior to loading on a gel. Proteins were separated on 12.5% SDS/polyacrylamide gels, blotted onto nitrocellulose, and detected using monoclonal 636 human lamin A/C antibody (1:50 diluted hybridoma cell supernatant)

(Rober et al., 1990), 346 mouse lamin A/C antibody (1:5 diluted) (Rober et al., 1989), monoclonal human vimentin V9 antibody (1:10,000 diluted) (Osborn et al., 1984), and monoclonal mouse vimentin V3 antibody (Osborn et al., 1984) (1:500 diluted) as described (Elbashir et al., 2002). Lamin A/C and vimentin antibody binding was either carried out simultaneously using both antibodies at the same time, or vimentin was detected after stripping the blot after lamin A/C detection. If stripping was carried out, the Western Blot recycling kit (Chemicon International, Temecula, CA) was used. Immunostaining was carried out using rabbit anti-mouse HRP-conjugated antibody (Dako Diagnostica, Hamburg, Germany) together with the Renaissance Western blot Chemiluminescence Reagent (NEN, Boston, MA). Quantitative analysis of chemiluminescent signals was performed using the Lumi-Imager system (Boehringer Mannheim, Germany) equipped with LumiAnalyst Version 3.0 software.

1.5 Real-time RT/PCR

After removal of the culture medium, A549 cells were lysed and the polyA mRNA was isolated using Sequitur's mRNA Catcher purification plate. RT-PCR analysis was performed using Sequitur's RT system, and each sample was measured in triplicate for the target gene and GAPDH. As primers for RT/PCR of lamin A/C the x and x were used. The percentage of inhibition is determined by the ratio of lamin A/C relative to GAPDH normalized to a non-specific siRNA control transfection.

2. RESULTS

2.1 Positional variation of siRNA targeting efficiency

Variations in the efficiency of gene silencing of siRNAs directed against different regions of an mRNA target have been reported previously, but were not investigated in a comprehensive or systematic manner. Our goal was to generate a large data set of siRNA targeting efficiencies directed against the abundant, non-essential nuclear lamina protein lamin A/C expressed in human HeLa SS6 and mouse SW3T3 cell lines. Lamin A (74

kD) and lamin C (65 kD) are isoforms generated by alternative splicing of the lamin A gene pre-mRNA and diverge only at their C-termini (Fisher et al., 1986; Lin and Worman, 1993; Machiels et al., 1996). Both isoforms are detected by a single monoclonal antibody 636 (Rober et al., 1990). The expression of lamin A/C is developmentally regulated (Stewart and Burke, 1987; Rober et al., 1989).

In a 24-well tissue culture plate format, siRNAs were delivered to the adherently growing cells by Oligofectamine transfection according to a standard protocol (Elbashir et al., 2002). Two or three days after transfection (depending on the experiment), lysates from cells were separated by SDS-PAGE and transferred on membranes for immunoblotting. The amount of lamin A/C was normalized to the unrelated protein vimentin (54 kD), which was also detected by a monoclonal antibody by probing the blot in parallel or after stripping of lamin A/C antibody. The efficiency of gene silencing is defined as the ratio of lamin A/C to vimentin signal in the presence of lamin A/C-specific siRNA relative to the ratio of lamin A/C to vimentin signal in the presence of GL2 luciferase control siRNA.

The mouse and human lamin A ORF and 3'-UTR sequences as well as the selected siRNAs are shown in a sequence alignment in Figure 2. The targeting site of an siRNA duplex is indicated by a bar. Mouse and human lamin A mRNAs are 87% identical and several larger segments of 100% identity are shared between both mRNAs. Sequence differences near these identity elements suggest that the RNA secondary structure of human and mouse lamin A/C mRNAs near those conserved segments may be different. This hypothesis was supported by RNA secondary structure predictions using mfold (Mathews et al., 1999; Zuker et al., 1999). With the exception of the targeting site for siRNA 1926, the selected identical targeting segments were folded in different secondary structures for mouse and human lamin A/C mRNAs. The targeting efficiency of 13 siRNAs (where 10

shared 100% identical targeting sites) was determined for endogenous lamin A/C in the human and mouse cell line. Representative Western blots used for quantification of targeted lamin A/C and control vimentin are shown in Figure 3A. The residual amount of lamin A/C protein was quantified 46 h after siRNA transfection and average values from at least three independent knockdown experiments are given in Figure 3B. Mouse lamin A/C was typically 2-fold less effectively reduced than human lamin A/C (Figure 3B). Nevertheless, siRNAs m607 and 1477 reduced mouse lamin A/C by more than 90%, indicating that near complete elimination of mouse lamin A/C was possible, and that the transfection efficiency was greater than 90% for both cell lines.

The general trend of less effective silencing in the mouse line suggests that sequence differences outside the targeted segments of human and mouse target mRNAs were not critical factors in explaining variations in targeting efficiency. The most effective siRNAs in human cells were also the most effective in mouse cells, and the least effective siRNAs in human cells were also the least effective in mouse cells and vice versa. This may suggest that the siRNA sequence by itself is recognized in a specific manner by the cell or that the target mRNA accessibility is not governed by the predicted overall secondary structure of the mRNA.

The effect of siRNAs containing mismatches when paired with the lamin A/C mRNA was also tested. Duplex m607 was specific for mouse, forming an A/G mismatch at position 16 (counting from the 5' end of the anti-sense guide siRNA) and a C/A mismatch at position 19 with the human target (Figure 2). Duplex h608 was specific for the human lamin A/C, forming a C/U mismatch at position 17 and a U/G wobble base pair at position 20 with the mouse target. Interestingly, although h608 effectively targeted both mouse and human lamin A/C, m607 only effectively targeted mouse lamin A/C. Presumably, U/G and C/U pairs are less disruptive than A/G and C/A pairs, and/or the mismatches occurring with h608 were one

nucleotide further downstream of the cleavage site than that occurring in m607. Inverting the G/C base pair at position 10 of the sense and anti-sense siRNA of h608, completely abolished human lamin A/C targeting. These few examples highlight that mismatched siRNA-guided target mRNA cleavage depends on the nature and the position of the mismatch with respect to the target mRNA.

In order to obtain a more comprehensive picture of the variation of siRNA targeting efficiencies, a total of forty-four different siRNA duplexes were directed against lamin A ORF or 3'-UTR in human HeLa cells. First, 25 siRNAs (including the siRNAs described before) were distributed at a distance of about 100 to 200 nucleotides covering the entire lamin A/C ORF and 3'-UTR (Figure 2). The C/G content of siRNAs varied between 32 (siRNA 2104) and 74 % (siRNA 1926). siRNAs were synthesized with dTdT 3'-overhangs whereby at least the penultimate dT of the antisense guide siRNA was fully complementary to the target mRNA (Elbashir et al., 2001c; Elbashir et al., 2002). When the lamin A/C content was measured 64 h post transfection, it was observed that all siRNAs had substantially reduced the levels of the target protein (Figure 4A). 23 of the 25 siRNAs had reduced lamin A/C expression by at least 75% and 11 out of 25 siRNAs reduced lamin A/C by more than 90%. The residual amount of protein detected in the knockdown cell populations can be accounted for by considering the sum of the non-transfected cells (showing no knockdown) and the residual lamin A/C protein within the knockdown cells. The most effective siRNAs were evenly distributed across the ORF and 3' UTR.

In order to evaluate whether the variations of siRNA efficiency observed in the experiments above are encountered at a similar frequency at a more local environment, we performed single-nucleotide walk experiments centered on the very effective siRNAs 778 and 781. siRNAs in that region were synthesized with only a single 2'-deoxythymidine residue at the 3'-

terminal position of the sense and antisense strand, all other 20 positions were unmodified and fully cognate to the target sequence. Only the first 20 nt of the antisense siRNA contribute to specificity of target mRNA recognition (Elbashir et al., 2001c). In this experiment, the incubation time of the cells after siRNA transfection was reduced from 64 h to 44 h in order to observe the knockdown differences before a plateau level was reached. The majority of siRNAs directed against this region reduced lamin A/C by more than 90% indicating that this entire region was very amenable to siRNA directed silencing. Nevertheless, 4 out of 21 siRNAs were clearly less effective than neighboring siRNAs, indicating that siRNA efficiency is not only influenced by local mRNA accessibility (Figure 4B). A similarly strong variation of targeting efficiency at a very local level is seen between siRNA 94 and 97 in human as well as mouse cells.

Finally, using siRNA 2148, which is specific for the lamin A isoform, we confirmed that a specific isoform can be depleted without affecting the level of lamin C (Figure 3A). Although we tested 3 different lamin C specific siRNAs, we were not able to specifically deplete lamin C (data not shown).

2.2 Comparison of duplex and single-stranded antisense siRNA targeting efficiencies

It was demonstrated recently that 5'-phosphorylated single-stranded antisense siRNAs could reconstitute RISC in cell lysates as well as in cell culture (Martinez et al., 2002a; Schwarz et al., 2002). However, only a few sequences were investigated for this property and the majority of previously published reports, which used mostly non-phosphorylated single-stranded antisense siRNAs, did not observe measurable silencing effects when compared to conventional duplex siRNAs. We therefore compared the previously tested 5'-phosphorylated antisense siRNA h608 (Martinez et al., 2002a) with the antisense siRNAs of the very effective siRNA duplexes 1053 and 1477 and the two moderately effective duplexes

447 and 817. The 5'-phosphate antisense silencing efficiencies were compared to those of 5'-phosphorylated and non-phosphorylated duplex siRNAs (Figure 5). As expected for duplex siRNAs, non-phosphorylated and 5'-phosphorylated siRNA duplexes behaved very similar because the non-phosphorylated siRNAs were rapidly phosphorylated by a cellular kinase (Nykänen et al., 2001; Martinez et al., 2002a). While the 5'-phosphorylated antisense siRNA h608 reduced lamin A/C expression by about 90%, other 5'-phosphorylated antisense siRNAs showed only a 20 to 40% reduction of lamin A/C.

Single-stranded RNAs are more susceptible to nuclease degradation than duplex siRNAs and the self-structure of the antisense siRNA strand may be important to contribute to its stability. We therefore examined the secondary structure of the 21-nt antisense siRNAs. No more than 4 contiguous base pairs can be formed internally. The antisense siRNAs h608 and 1053 could form the most stable stem-loops, and siRNA 1477 could not form a stable stem-loop. The stem-loop of siRNA h608 was positioned at the 3'-end only exposing the 2'-deoxythymidine residues, which may provide substantial protection against 3'-exonucleases, that are predominantly responsible for small RNA degradation. For the other antisense siRNAs, the predicted stem-loops were located more centrally. Beside a self-structure, we also noticed that antisense siRNA h608 has a significant potential for forming a self-dimer, where a helix with 3-nt 3'-overhangs could form containing a total of 12 regular base pairs, 2 G/U wobble base pairs and 2 C/U and 2 U/U mismatches. In comparison, siRNA 1053 has the potential of also forming a self-dimer (i. e. a duplex) with a 2-nt 3' overhang, and a total of 10 base pairs and 9 mismatches. A small fraction of siRNA h608 folded into the predicted secondary structures may be sufficient to explain its more favorable entering into the RNAi pathway in comparison to the other siRNAs.

2.3 Conjugation of fluorophores to siRNAs

One of the challenges of siRNA-based gene silencing is to obtain homogenous pools of knockdown cells, which is currently only feasible by using cells that are readily transfected, such as HeLa, A549, or SW3T3 cells. To separate knockdown cells from non-transfected cells, fluorescently labeled siRNAs could probably be used to separate transfected cells from non-transfected cells by fluorescence-activated cell sorting (FACS). We therefore tested what positions of the sense and the antisense siRNA could be fluorescently labeled without compromising its activity and whether uptake of fluorescently labeled siRNAs could be correlated with gene silencing. Fluorescein was conjugated to lamin A/C siRNA duplex h608 at the 5′ or 3′ ends of the sense or antisense siRNA strand. The fluorescein chromophore was separated by a 6-carbon linker from the 5′-phosphate and by a 3-carbon/amide bond/5-carbon linker from the 3′-phosphate. As non-specific siRNA control, luciferase sense siRNA GL2 was also modified at its 5′ end. Immunofluorescence analysis of lamin A/C knockdown (Elbashir et al., 2002) indicated that modification of the sense strand at the 5′ or 3′ end did not interfere with gene targeting, while modification of the antisense strand was tolerated only at its 5′-end (Figure 6). The tolerance for 5′ modifications at the antisense siRNA was not anticipated because recent biochemical or cell biological analysis of the mechanism of siRNAs indicated that a free terminal 5′-phosphate was essential (Nykänen et al., 2001; Chiu and Rana, 2002; Martinez et al., 2002a; Schwarz et al., 2002). It was also unexpected that our modification of the antisense 3′-end with fluorescein or Alexa488 did abolish RNAi, because it was reported that the introduction of a 3-carbon aminolinker, a 3′-puromycin or 3′-biotin did not interfere with gene silencing (Chiu and Rana, 2002). Furthermore, it was also shown that a 3′-fluorescein label that was linked via a 6-carbon linker and a thiourea bridge to the fluorophore antisense siRNA did hardly compromise its activity (Holen et al., 2002).

Most interestingly, we find that uptake of fluorescently labeled siRNAs was not always correlated with gene silencing, and that small foci of concentrated fluorescent dyes, most likely representing endosomal compartments, are also detected in cells that do not show lamin A/C silencing (indicated by white arrows in Figure 6). Near 100% fluorescent labeling of transfected cells was also observed previously (Holen et al., 2002), but no correlation of uptake and silencing was made using single cell immunofluorescence.

To monitor the distribution of the Alexa488-modified siRNAs after Oligofectamine transfection into HeLa cells, we used confocal microscopy. Twenty hours after transfection the siRNAs were typically found localized to discrete foci on the cytoplasmic side of the nuclear membrane (Figure 7). Similar endosomal localization patterns in the cytoplasm have been observed after uptake of fluorescent-conjugated phosphorothioate antisense oligonucleotides (Bennett et al., 1992; Hartmann et al., 1998). However, phosphorothioate antisense oligonucleotides also show diffuse nuclear staining when transfected with cationic carrier lipids and such nuclear localization has been correlated with increased antisense efficiency (Bennett et al., 1992). Nuclear localization of phosphorothioate antisense oligonucleotides is apparently due to thiophosphate-mediated association of the liposome/endosome-released antisense oligonucleotide with some nuclear import factor.

2.4 Chemical modification of the sugar-phosphate siRNA backbone

Modification of the sugar-phosphate backbone of DNA and RNA molecules influences the stability and pharmacological properties of antisense molecules (Verma and Eckstein, 1998; Jen and Gewirtz, 2000; Eckstein, 2002). We wanted to examine if standard modifications, such as thiophosphate and 2'-fluoro, were compatible with the recognition of the siRNA duplex by the cellular RNAi machinery. Using our effective mouse lamin A/C siRNA m607 as reference point, we introduced thiophosphate

linkages at the 5'-most internucleotidic linkage, ps(2), at the three 3'-most internucleotidic linkages, ps(19-21), or at every second position, ps(even#) (Figure 8A). Unmodified and modified siRNAs strands were annealed in the various combinations shown in Figure 8B and tested for lamin A/C knockdown in mouse SW3T3 cells. The efficiency of silencing for all these duplexes was comparable strong, but siRNA duplexes with 50% thiophosphate content showed cytotoxic properties and reduced cell growth and viability as determined by cell counting (data not shown).

We also tested the effect of gene silencing of 3'-terminal thiophosphate-modified human lamin A/C siRNA h608 (Figure 8D), and obtained similar results. Thiophosphate modifications did not alter the gene-silencing properties of h608 siRNA in HeLa cells and antisense siRNA h608 alone was also able to silence lamin A/C with comparably high efficiency than the unmodified antisense siRNA (Figure 8E).

Besides thiophosphate modifications, sugar-modifications are also used to stabilize RNAs against nucleases (Verma and Eckstein, 1998). One of the more prominent and commercially available RNA analogs are the 2'-fluoro-2'-deoxyribnucleotides. Substitution of only the pyrimidine residues is sufficient to substantially stabilize the RNA against endonucleases. We replaced the C and U residues of human lamin A/C 778 duplex with 2' fluoro-2'-deoxyuridine and 2'-fluoro-2'-deoxycytidine, respectively (Figure 9A). The 21-nt antisense siRNA of this duplex is almost entirely composed of pyrimidines and contains 15 2'-fluoro-2'-deoxy-U and -C nucleotides and two 2'-deoxythymidine residues. We also tested the 3'-terminal thiophosphate modifications for duplex 778 as well as the combination of thiophosphate and 2'-fluoro-2'-deoxyribose. All of these modified siRNA duplexes were indistinguishable from the unmodified standard siRNA duplex (Figure 9B). Furthermore, we did not notice any unspecific toxicity of these backbone modifications when comparing the target-nonspecific 2'-

fluoro-2'-deoxy pyrimidine and 3'-terminal thiosphosphate-modified GL2 siRNA to unmodified GL2 (data not shown).

Nuclease-resistant antisense oligonucleotides are known to enter cells without transfection. We therefore provided the modified, nuclease-resistant siRNA duplexes in the tissue culture medium without encapsulation by transfection reagents, hoping that siRNA duplexes may enter the cell via the endocytosis pathway. However, even at 1 $\mu$M siRNA duplex concentrations in the tissue culture medium, we were unable to induce lamin A/C gene silencing (data not shown).

2.5 RNA hairpin-based human lamin A/C gene silencing

RNA hairpins are double-helical structures where the 5' and 3' end of a helical terminus is joined by a short single-stranded loop. These stem-loop structures resemble the precursors of microRNAs that also effectively enter the RNAi pathway (Hutvágner and Zamore, 2002; Llave et al., 2002). Similar to the processing of long dsRNA to siRNAs, RNA hairpins are processed by Dicer RNase III to generate siRNA or miRNAs (Bernstein et al., 2001; Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001). Many hairpin constructs have been described for gene silencing, differing in stem length, loop orientation and loop sequence. We wanted to systematically evaluate the contribution of the loop orientation and length of the stem. As outlined before, the position of target RNA cleavage can have a profound impact on silencing efficiency, and variation of the stem length and loop orientation would invariably affect targeting position. We therefore directed synthetic hairpin RNAs against the lamin A/C region at position 770 to 810 that was scanned before with siRNA duplexes in single-nucleotide intervals (Figure 4B).

The length of the stem was varied in 2 base pair intervals from 19 to 29 nt, and a 2-nt dTdT 3'-overhang was added on one side while a UUCG-tetraloop connected the 5' and 3' ends on the other side of the stem

(Figure 10A). In one subset, the 3' end of the sense strand was joined to the 5' end of the antisense strand (indicated by R, right). In the other subset, the 3'-end of the antisense strand was joined to the 5' end of the sense strand (indicated by L, left). Because processing of dsRNA by Dicer was shown to occur predominantly from the ends of the dsRNA (Zhang et al., 2002), it might be assumed that the L constructs do not change the targeting site while the R constructs change the site according to the number of inserted base pairs. Our preceding analysis of the cleavage efficiencies of siRNAs of that region indicated that only the 27 and 29 base pair R-type construct could show reduced cleavage efficiency, and only if Dicer processing would produce an siRNA identical to siRNA 786 (Figure 4B). The processing of hairpins may also be influenced by sequence-specific preferences of Dicer as it was noticed previously that siRNA and miRNA termini preferentially begin with a 5' uridine (Elbashir et al., 2001b; Lau et al., 2001), thus implying a preference for cleaving at uridine residues.

Quantification of siRNA knockdown was performed by two independent methods, either by monitoring the amount of residual lamin A/C protein by quantitative immunoblotting (Figure 10B) or the residual amount of lamin A/C mRNA using real-time RT/PCR (Figure 10D). The first indications of lamin A/C protein reduction are observable about 24 h past transfection. The analysis of residual lamin A/C protein was performed 44 h past siRNA transfection in HeLa cells, at a time point where silencing is readily detectable but has not yet reached its maximum value. This time point was selected to better observe the differences in efficiency between the various duplexes and hairpins tested. With the exception of the 19 base pair R19A construct, all hairpins performed comparably and effectively suppressed lamin A/C expression similarly if not slightly better than the classic duplexes tested before. The relative variation in lamin A/C h608 and 778 gene silencing between Figure 10B and the previous Figure 4B are best explained by batch to batch variation of the Oligofectamine transfection

reagent and HeLa cells. The averaging of data series for Figure 10B was independent from previous experiments that were performed several months in the past. The differences in silencing efficiency between siRNA duplexes and hairpin RNAs appears to reflect a small difference in kinetic rates of entering RISC, because at a later time point, at 64 h past transfection, a difference in silencing between siRNAs and hairpins, with the exception of R19A, has become undetectable (data not shown). Hairpin R19A was still less effective than any other hairpin or siRNA duplex, indicating that 19 base pair stems with a UUCG loop on the 5' end of the antisense siRNA strand are suboptimal.

The reduction of mRNA levels correlated well with the residual protein levels.

## 3. DISCUSSION

### 3.1 Factors affecting siRNA targeting efficiency

The design of a siRNA-based knockdown experiment requires the selection of a targeting region within the target mRNA that is (1) unique in sequence in the genome of the studied organism and (2) readily cleaved by the siRNA-targeting complex. Currently, the identification of specific siRNAs is very much a trial and error process. The specificity of the knockdown is generally controlled by analyzing the knockdown phenotype of several siRNAs that are unrelated in sequence but cognate to different segments of the targeted mRNA. The selection of specific siRNAs can be partially improved by using sequence analysis software such as NCBI Blast for identifying closely related sequences in the genome databases that could be mistargeted by the selected siRNAs. However, certain non-Watson-Crick base pairs (Leontis et al., 2002) that should not perturb helical geometry within a siRNA/target mRNA complex are not recognized by such algorithms. Furthermore, siRNA may also display features of miRNAs, which act as translational repressors in animal systems, in that a selected

siRNA may recognize a non-cognate mRNA if a central bulge similar to a miRNA-specific targeting complex could be formed (Ambros, 2001).

Besides the specificity, the efficiency of siRNA-mediated gene silencing is another important factor, and rules to predict the efficiency of siRNAs have not yet been developed. A large systematic analysis has not yet been performed, probably because sufficiently accessible site are readily identified for the targets studied that far (Harborth et al., 2001). In order to assist the development of genome-wide sets of specific and potent siRNA duplexes, we wanted to explore the variations associated with changes in the targeting sites. The efficiency of silencing may be affected by many parameters, some of which we have tried to dissect by targeting a homologous gene expressed in mouse and human cells.

We observed that global differences in the response to transfected siRNAs exist between human HeLa cells and mouse SW3T3 cells by selecting siRNAs that recognize both human and mouse lamin A/C. The reduction of lamin A/C was generally less efficient in SW3T3 cells even though several siRNAs could be identified that reduced mouse lamin A/C to more than 90%. Thus, the differences in reduction of mouse lamin A/C were not due to differences in transfection efficiency between mouse and human cells, and rather suggest that a cellular factor involved in RNAi is limiting. The behavior of SW3T3 cells is similar to those of human keratinocyte HaCaT cells, where the identification of powerful siRNAs targeting human coagulation trigger tissue factor was more difficult (Holen et al., 2002) than we would have predicted from our analysis using HeLa cells (Harborth et al., 2001). Such global differences suggest cell-type specific differences in expression of general mRNA binding proteins reducing accessibility by RISC, or by reduced concentration of one or several protein factors required for formation of RISC. Alternatively, high cell type-specific expression levels of endogenous microRNAs (Lagos-Quintana et al., 2002) may compete with transfected siRNAs for the cellular RISC-constituting

Argonaute proteins eIF2C1 and eIF2C2 (Hutvágner and Zamore, 2002; Martinez et al., 2002a; Mourelatos et al., 2002). Competition of active siRNAs by inactive siRNAs as been shown previously (Holen et al., 2002). Establishment of stable cell lines that express increased levels of such proteins could probably overcome such limitations and boost genome-wide functional screening analysis.

Prediction of secondary structure of target mRNAs does not appear to be helpful in predicting siRNA targeting efficiency. Although the predicted lamin A/C secondary structure of lamin mouse and human lamin A/C were different, we found that siRNAs directed against shared sequences showed a very similar efficiency profile in human and mouse cell lines. It is therefore proposed that the siRNA sequence itself rather than the target RNA structure is the key determinant. This hypothesis is also supported by the single nucleotide interval siRNA walk, where it was found that a single siRNA, despite the flanking of very effective siRNAs on both sides can be reduced in targeting efficiency (Figure 4B).

Finally, the quality of the cDNA sequence used for siRNA design is critical. We were aware of the possibility of single-nucleotide polymorphisms (SNPs) or sporadically arising mutations in transformed cell lines and screened databases for SNPs. Four SNPs were annotated in the cDNA sequence of human lamin A (Figure 2). In addition, we also cloned and sequenced the lamin A gene from our SW3T3 and HeLa cells. Six and seventeen single-nucleotide changes were found for mouse and human lamin A with respect to the database entries for mouse and human lamin A, respectively. None of the SNP sites or the mutations fell into any of the targeted regions. These examples indicate how critical it is that a reliable mRNA sequence is used for the design of siRNAs.

3.2 Mismatch targeting

The effect of mismatches on specificity of target RNA cleavage is not only dependent on the position of the mismatch relative to the target RNA cleavage site but also on the geometry and probably also the thermodynamic stability of the non-Watson-Crick interaction. It was shown previously that the first 20 nucleotides of the guide antisense siRNA contributed to specificity, and that only the 3' most nucleotides of the antisense siRNA (position 21) did not (Elbashir et al., 2001c). It is however conceivable that non-canonical base pairs, for example those that are frequently found in double-stranded microRNA precursors (e.g. G/U, U/G, C/A, A/C, U/U, C/U, U/C) (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001), are tolerated to some extent during siRNA-mediated target recognition. Evidence for such non-Watson-Crick pairing during target recognition was provided in Figure 3, where double mismatches of different sequence were tolerated in one case but not the other. Single mismatches positioned in the center of an siRNA guide strand can confer complete specificity, as was shown here, were a C/C mismatch was not tolerated in the mRNA targeting complex. Near complete discrimination between mismatched and matched target RNAs was also described during siRNA targeting of point-mutated oncogenes (Brummelkamp et al., 2002a; Martinez et al., 2002b).

3.3 Isoform-specific targeting

One of the advantages of post-transcriptional gene silencing is the ability to specifically target spliced mRNA isoforms (Smith and Valcarcel, 2000). Isoforms sometimes encode proteins with drastically different function even though all are derived from one gene locus. By directing an siRNA against a sequence portion unique to one of the isoforms, it was effectively depleted, as shown for lamin A (Figure 3A). Specific targeting of lamin A without affecting lamin C expression also indicates the absence of transitive RNAi, a phenomenon observed in C. elegans (Sijen et al., 2001) and plants (Klahre et al., 2002; Vaistij et al., 2002), but not detectable in

mammalian cell lines. Transitive RNAi is caused by spreading of silencing into sequences adjacent to the targeted region, and requires dsRNA synthesis by putative RNA-dependent RNA polymerases. Biochemical evidence against spreading in mammalian and Drosophila systems was recently provided (Chiu and Rana, 2002; Martinez et al., 2002a; Schwarz et al., 2002). In a different approach, silencing of several isoforms was accomplished by directing a siRNA against a sequence segment shared by all isoforms (Kisielow et al., 2002). Concomitantly with the knockdown, it was shown to be possible to ectopically express one of the isoforms from a plasmid carrying the isoform-specific cDNA with a silent mutation at the siRNA targeting site (Kisielow et al., 2002). In mammalian systems, the high efficiency of siRNA-mediated gene silencing is best explained by the catalytic nature of the RISC complex, which is able to catalyze the degradation of more than one mRNA molecule (Hutvágner and Zamore, 2002).

3.4 Chemically modified siRNAs

Conjugation of fluorophores to siRNAs is an effective means of visualizing siRNAs by fluorescence microscopy or fluorescence spectroscopy after transfection into cells or injection into organisms. Caution has to be taken, however, when a correlation between localization and activity of the siRNA is to be made. This is illustrated by our observation that transfected fluorescent siRNA duplexes, which were detect as small foci (most likely endosomes) by fluorescence microscopy inside almost all cells, did show target gene silencing within all fluorescent cells. Presumably, not all endosomes release the siRNAs in an effective manner. If fluorescence activated cell sorting (FACS) is used to separate transfected from non-transfected cells it is important to note that some of the sorted cells may still escape silencing. Other delivery modes, such as electroporation or calcium phosphate precipitation may be tested and may prove more reliable for FACS sorting of siRNA-containing cells.

Introduction of phosphorothioate and 2'-fluoro-2'-deoxyribose substitutions is an interesting approach to enhance or modify the pharmacological properties of siRNAs and does not affect the activity of siRNAs. These modified siRNAs now need to be tested in animal models if they provide any advantages over the conventional siRNAs. Phosphorothioate and 2'-fluoro-2'-deoxyribose modifications were previously incorporated enzymatically into long dsRNAs that were then injected into C. elegans (Parrish et al., 2000). Consistent with our finding, it was shown that 2'-fluoro-2'-deoxyuridine-modified dsRNA behaved identical to unmodified dsRNA. Phosphorothioate-modification of one of the four nucleotide 5'-phosphodiester linkages dsRNA did only mildly compromise activity when the antisense strand was modified by uridine 5'-thiophosphates. The latter effect may be a consequence of reduced dsRNA processing because Dicer RNase III, which appears to preferentially cleave after 5'-pyrimidine residues (Elbashir et al., 2001b; Lau et al., 2001). The toxicity of highly thiophosphate-substituted siRNAs was not observed in C. elegans experiments because only one of the 4 nucleotides was replaced at a time (Parrish et al., 2000). In a recent report targeting of HIV-1 gag mRNA (Capodici et al., 2002), it was demonstrated that transcribed 2'-fluoro-2'-deoxyribo-C- and -U-modified siRNAs that carry a 5'-terminal triphosphate could be delivered to cells without lipofectin transfection and in the presence of serum. The differences between our findings may be explained by differences in the cell lines or by the presence of a 5'-triphosphate rather than a 5'-monophosphate at the siRNA. It is conceivable that 5'-triphosphates may be recognized by some cell surface receptor that facilitates cellular uptake of the siRNA duplex. Also, D. melanogaster Schneider 2 cells can take up long transcribed dsRNA added directly to the tissue culture medium, indicating that specific cell lines exist that take up dsRNA without transfection.

3.5 Hairpin siRNAs

Gene suppression data for lamin A/C siRNAs and hairpin RNAs were obtained by measuring residual lamin A/C protein as well as residual intact target mRNA. Even though two different human cell lines are compared by two different methods, the residual amount of mRNA correlated well with the residual amount of protein. It is important to carefully select the primer sites and siRNA targeting sites if it is anticipated that the knockdown has to monitored by RT/PCR. Preferably, the primer pairs are positioned far upstream of the siRNA cleavage site because 5' mRNA cleavage products (that do not have a poly(A)tail) are readily removed by poly(A)-based solid phase mRNA preparation method and cannot perturb the analysis. Depending on the sequence and secondary structure of the mRNA cleavage fragments, they may remain stable even though they are no longer translated.

3.6 Enhancing specificity of siRNAs

siRNAs duplexes contain a sense and an antisense strand, only one of which is required for target mRNA recognition. The specificity of gene targeting may be enhanced if mistargeting by siRNA duplexes is minimized. It is therefore proposed to inactivate the strand that is non-complementary to the target mRNA, for example by introducing a non-nucleotidic 5' or 3' modification that interferes with RISC formation of that strand. Alternatively, and this may be much more elegant, it is suggested to identify targeting sites that are (partially) palindromic over a region of approximately 20 nucleotides. Only one single siRNA strand needs to be synthesized that can anneal to itself. Therefore, both strands of the siRNA duplex would contribute to recognition of the same targeting site. This approach would also reduce production costs while increasing the specificity. Many examples of non-Watson-Crick base pairs compatible with the selection of partially palindromic sequences are found in the double-stranded hairpin precursor sequences of miRNAs.

In summary, we have shown that targeting sites accessible to siRNAs are also effectively targeted by short hairpin RNAs. Synthetic hairpin RNAs with chemical linker loops may become a more cost-effective alternative to synthetic siRNAs that are normally composed of two single-strands.

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## Claims

1. A double-stranded RNA molecule wherein each RNA strand has a length of from 15-50 nucleotides comprising an antisense strand which is substantially complementary to a target transcript and a sense-strand which is at least substantially complementary to the antisense strand and wherein the RNA molecule has an at least partially palindromic sequence

2. The RNA molecule of claim 1 having at least one 5'- and/or 3' overhang.

3. The RNA molecule of claims 1 or 2 having at least one 3' overhang.

4. The RNA molecule of any one of claims 2-3 wherein the overhang length is from 1-5 nucleotides, particularly from 1-3 nucleotides.

5. The RNA molecule of any one of claims 1-4 having free 5' hydroxyl moieties and/or moities selected from phosphate groups or analogues thereof.

6. The RNA molecule of any one of claims 1-5 wherein the palindromic sequence has a length of from 10-30 nucleotides.

7. The RNA molecule of any one of claims 1-6 wherein the antisense strand has a portion which is completely complementary to the target transcript.

8. The RNA molecule of any one of claims 1-7 wherein the palindromic sequence has a self-complementarity of at least 80% based on the number of nucleotides in the palindromic sequence.

9. The RNA molecule of any one of claims 1-8 wherein the antisense strand is completely complementary to the sense strand.

10. The RNA molecule of any one of claim 1-8 wherein the antisense strand is not completely complementary to the sense strand.

11. The RNA molecule of any one of claims 1-10 wherein the complementary base pairs between the antisense and sense strands include preferred complementary base pairs selected from A/U, U/A, G/C, C/G, G/U and U/G base pairs and less preferred complementary selected from U/U, C/U, U/C, A/A, and G/G.

12. The RNA molecule of claim 11 wherein the proportion of less preferred complementary base pairs of the total complementary base pairs is not more than 50%.

13. The RNA molecule of any one of claims 1-12 wherein the sequence of the antisense strand is identical to the sequence of the sense strand.

14. A double-stranded RNA molecule wherein each RNA strand has a length of from 15-50 nucleotides comprising an antisense strand which is at least substantially complementary to a target transcript and a sense strand which is at least substantially complementary to the antisense strand and wherein the RNA molecule carries at least one labelling group on the 5'- or 3'-end of the sense strand or on the 5'-end of the antisense strand.

15. The RNA molecule of claim 14 wherein the labelling group is coupled via a linker.

16. The RNA molecule of claim 15 wherein the linker has a chain length of 4-20 atoms.

17. The RNA molecule of any one of claims 14-16 wherein the labelling group is a fluorophore.

18. A double-stranded RNA molecule wherein each RNA strand has a length from 15-50 nucleotides comprising an antisense strand which is at least substantially complementary to a target transcript and a sense strand which is at least substantially complementary to the antisense strand and wherein the sense strand comprises a non-nucleotidic 5'- and/or 3'-modification which inhibits RNA interference.

19. The RNA molecule of any one of claims 1-18 comprising at least one modified nucleotide analogue.

20. The RNA molecule of claim 19 wherein at least one modified nucleotide analogue is selected from phosphate-, sugar- and nucleobase-modified analogues and combinations thereof.

21. The RNA molecule of claim 20 wherein the at least one modified nucleotide is a 2' fluoro-modified ribonucleotide analogue.

22. The RNA molecule of claim 21 wherein the at least one modified nucleotide is a phosphorothioate nucleotide analogue.

23. A double-stranded RNA molecule wherein each RNA strand has a length from 15-50 nucleotides comprising an antisense strand which is at least substantially complementary to a target transcript and a sense strand which is at least substantially complementary to the antisense strand and wherein at least one strand comprises at least

one 2'-OH modified nucleotide and at least one modified phosphoester group.

24. The RNA molecule of claim 23 comprising at least one 2'OH modified pyrimidine nucleotide.

25. The RNA molecule of claim 23 or 24 comprising up to 20 2'OH modified nucleotides.

26. The RNA molecule of any one of claims 23-25 wherein the 2'OH modified nucleotide is a 2' fluoro nucleotide.

27. The RNA molecule of any one of claims 23-26 comprising up to 10 modified phosphoester groups.

28. The RNA molecule of any one of claims 23-27 wherein the 3' terminal phosphoester groups are modified.

29. The RNA molecule of any one of claims 23-26 wherein the modified phosphoester group is a phosphorothioate group.

30. The RNA molecule of any one of claims 1-29 for the inhibition of target gene expression in vitro.

31. The RNA molecule of any one of claims 1-29 for the inhibition of target gene expression in vivo.

32. A pharmaceutical composition comprising at least one RNA molecule of any one of claims 1-31 as an active ingredient and pharmaceutically acceptable carriers, diluents and/or adjuvants.

33. The composition of claim 32 which is a cationic liposomal or lipid formulation.

34. The composition of claims 32 or 33 for diagnostic applications.

35. The composition of claim 34 for the monitoring of diseases associated with overexpression of at least one target manuscript.

36. The composition of claims 32 or 33 for therapeutic applications.

37. The composition of claim 36 for the prevention or treatment of diseases associated with overexpression of at least one target manuscript.

38. The composition of claims 35 or 37 wherein the diseases are selected from tumour diseases, inflammatory diseases, infectious diseases, e.g. viral infections, degenerative diseases and autoimmune diseases.

Figure 1


9-nt
A
mRNA 5′...NNNAAUGCUUAAGUCUUAAGCAUNNN...
AUGCUUAAGACUUAAGCAUTT as siRNA
TTUACGAAUUCAGAAUUCGUA as siRNA
B
mRNA 5′...NNNAAUGCUUGAGUCUUAAGCAUNNN...
AUGCUUAAGACUCAAGCAUTT as siRNA
TTUACGAACUCAGAAUUCGUA as siRNA
C
mRNA 5′...NNNAAUGCUUAAAUCUUAAACAUNNN...
AUGUUUAAGAUUUAAGCAUTT as siRNA
TTUACGAAUUUAGAAUUUGUA as siRNA
D
mRNA 5′...NNNAAUGCUGAAGUCUUAAGCAUNNN...
AUGCUUAAGACUUCAGCAUTT as siRNA
TTUACGACUUCAGAAUUCGUA as siRNA
E
mRNA 5′...NNNCAUGCUUAAGUCUUAAGCAUNNN...
AUGCUUAAGACUUAAGCAUGT as siRNA
TGUACGAAUUCAGAAUUCGUA as siRNA

## Figure 2

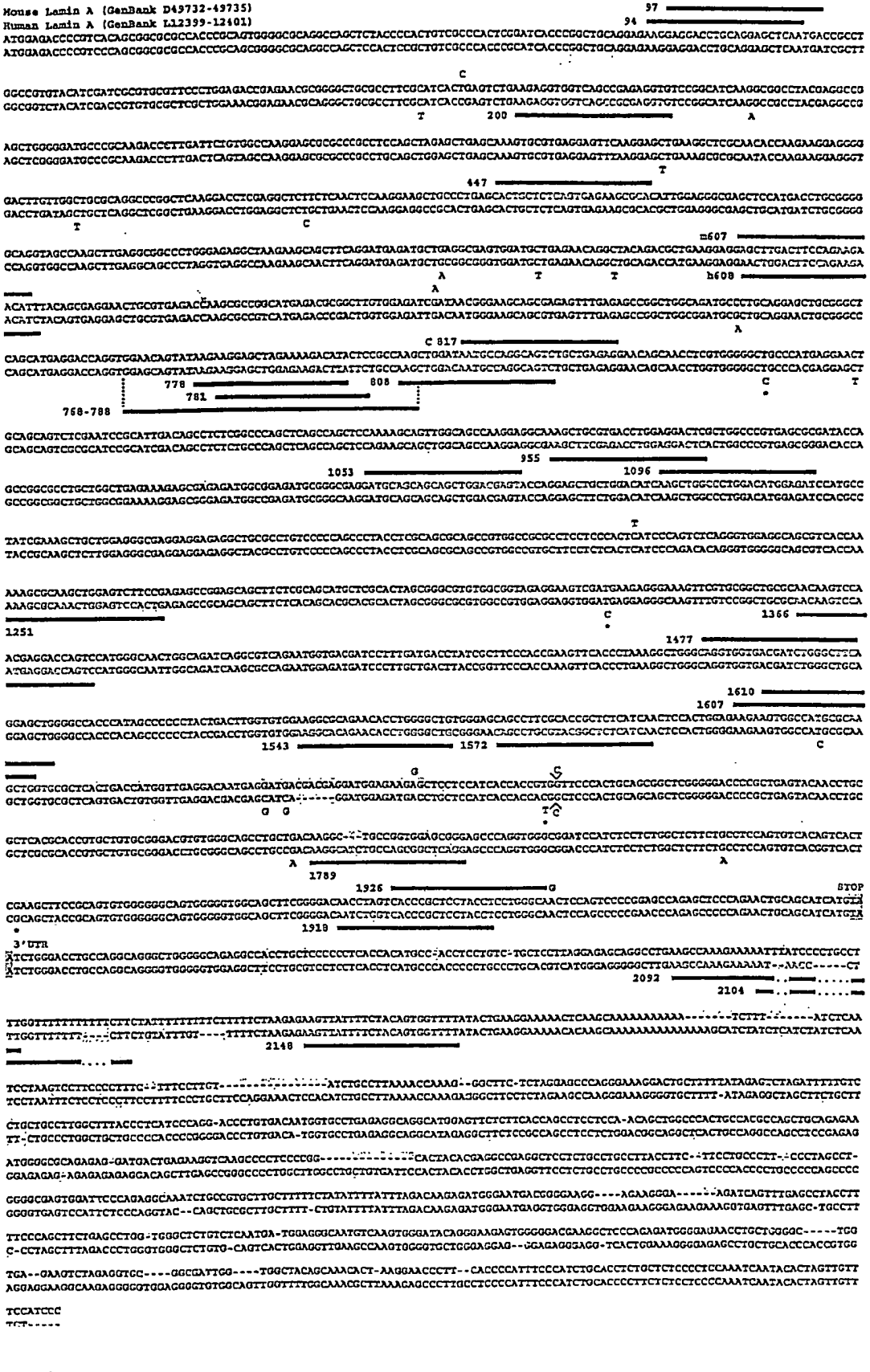

Mouse Lamin A (GenBank D49732-49735)
Human Lamin A (GenBank L12399-12401)
97
94
200
447
m607
h608
C 817
778
808
781
768-788
955
1053
1096
1366
1251
1477
1610
1607
1543
1572
1789
1926
STOP
1918
3'UTR
2092
2104
2148

Figure 3

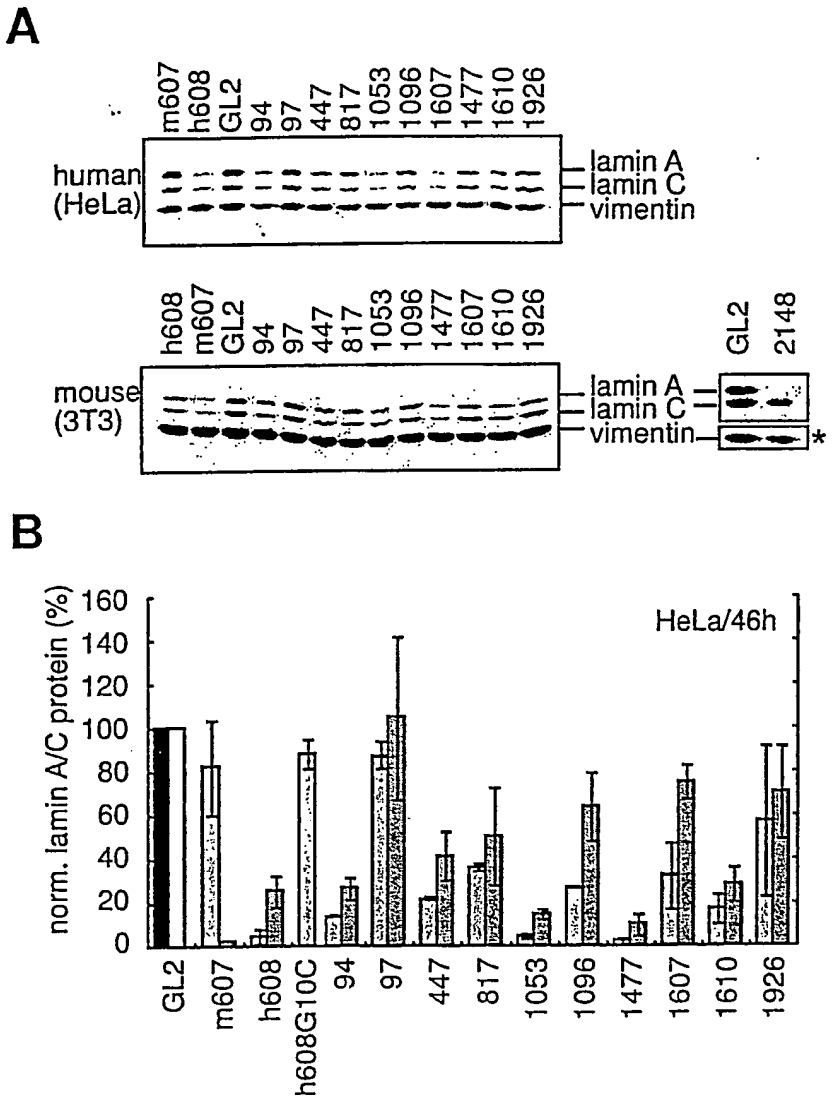

A
m607
h608
GL2
94
97
447
817
1053
1096
1607
1477
1610
1926
human
(HeLa)
lamin A
lamin C
vimentin
h608
m607
GL2
94
97
447
817
1053
1096
1477
1607
1610
1926
GL2
2148
mouse
(3T3)
lamin A
lamin C
vimentin
*
B
norm. lamin A/C protein (%)
160
140
120
100
80
60
40
20
0
HeLa/46h
GL2
m607
h608
h608G10C
94
97
447
817
1053
1096
1477
1607
1610
1926

Figure 4

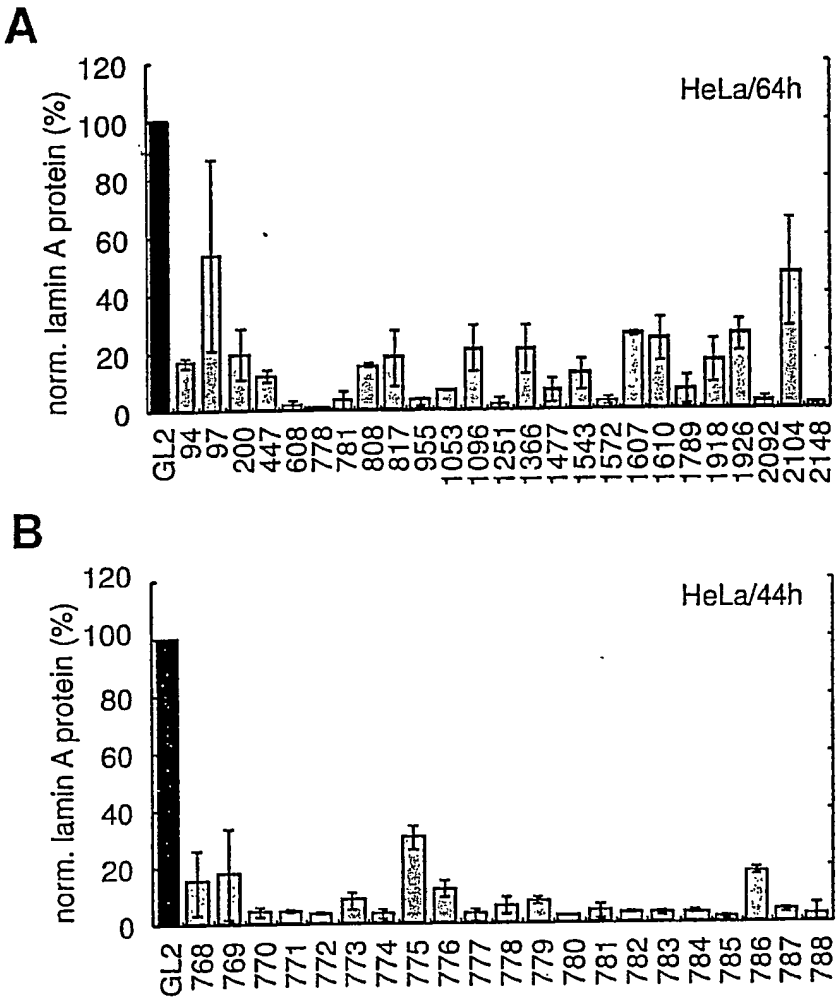
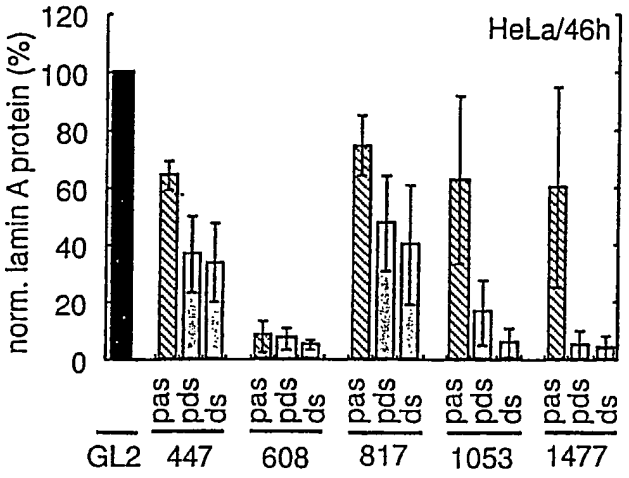

A
HeLa/64h
norm. lamin A protein (%)
120
100
80
60
40
20
0
GL2
94
97
200
447
608
778
781
808
817
955
1053
1096
1251
1366
1477
1543
1572
1607
1610
1789
1918
1926
2092
2104
2148
B
HeLa/44h
norm. lamin A protein (%)
120
100
80
60
40
20
0
GL2
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788

Figure 5


HeLa/46h
norm. lamin A protein (%)
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100
80
60
40
20
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pas
pds
ds
GL2
447
608
817
1053
1477

Figure 6A

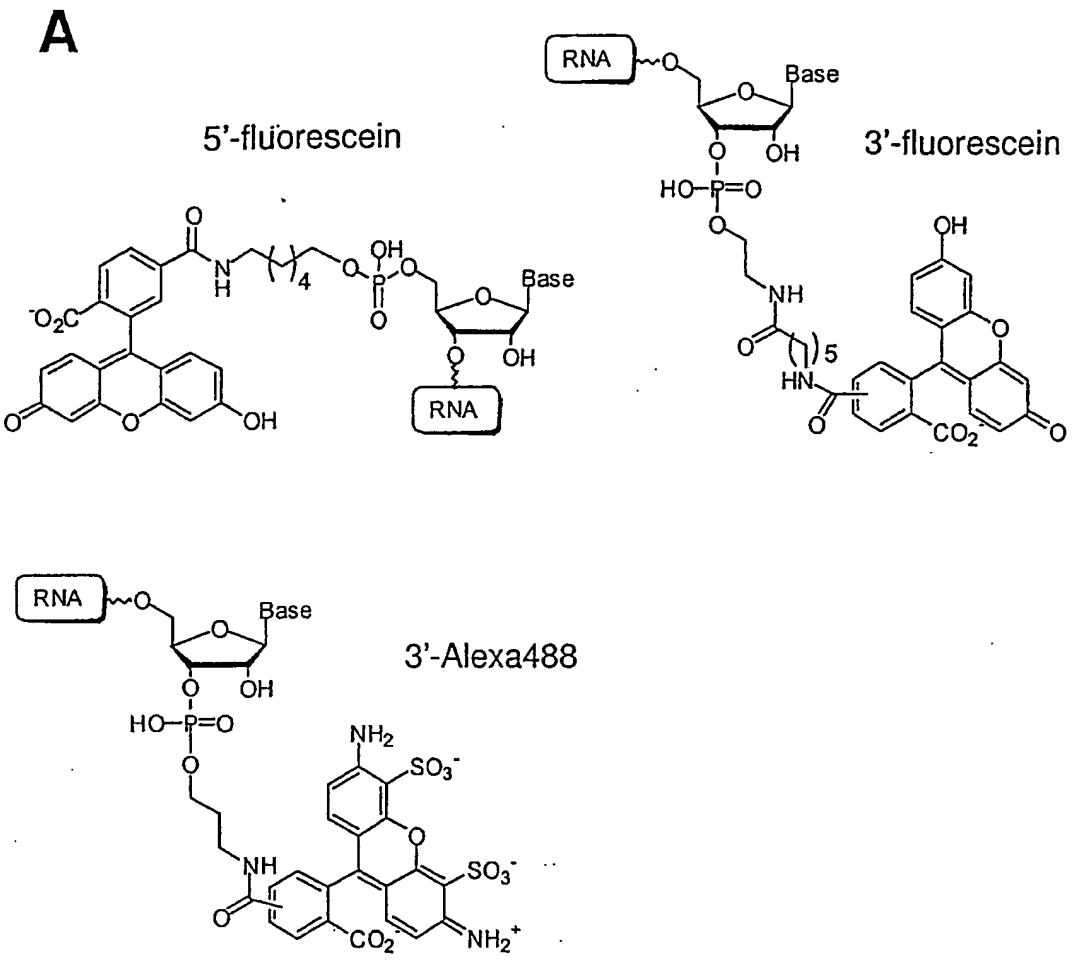

A
5'-fluorescein
3'-fluorescein
3'-Alexa488
RNA
Base
OH
HO–P=O
NH
HN
RNA
Base
OH
HO–P=O
NH
NH2
SO3-
SO3-
CO2-
NH2+

Figure 6B

B

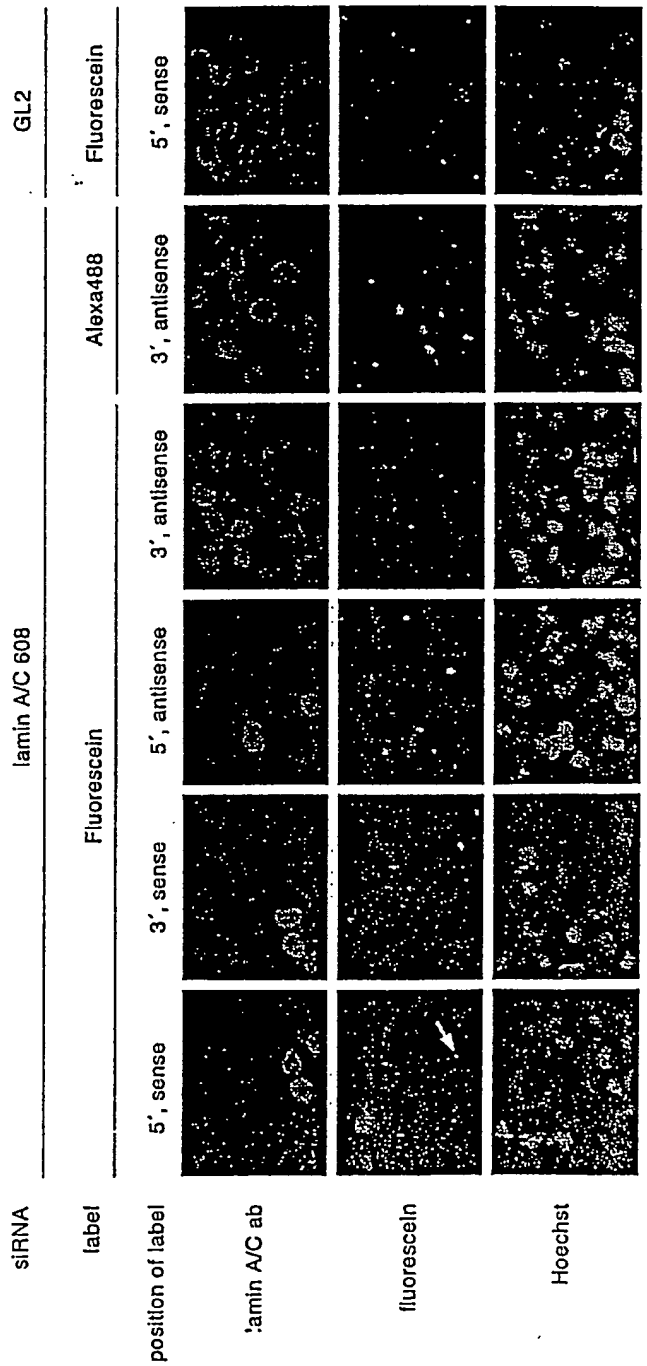

siRNA
lamin A/C 608
GL2
label
Fluorescein
Alexa488
Fluorescein
position of label
5', sense
3', sense
5', antisense
3', antisense
3', antisense
5', sense
lamin A/C ab
fluorescein
Hoechst

Figure 7

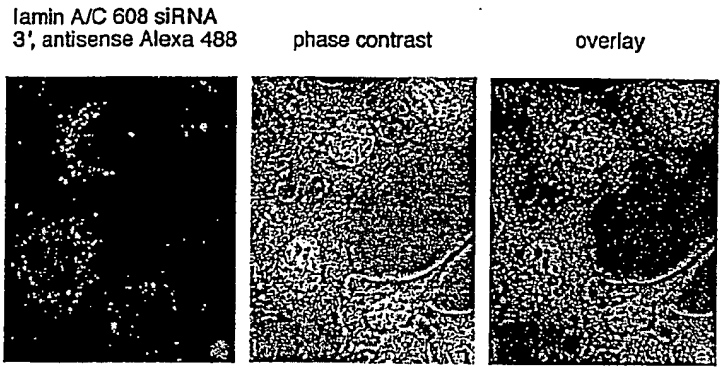

lamin A/C 608 siRNA
3', antisense Alexa 488
phase contrast
overlay

Figure 8 A,B

A

mouse target region (607-629)
AAGCUUGACUUCCAGAAGAACAU

| | |
|---|---|
| s | GCUUGACUUCCAGAAGAACTT |
| s-ps(even#) | GpsCUpsUGpsACpsUUpsCCpsAGpsAApsGApsACpsTT |
| s-ps(19-21) | GCUUGACUUCCAGAAGAApsCpsTpsT |
| s-ps(2) | GpsCUUGACUUCCAGAAGAACTT |
| GL2 s | CGUACGCGGAAUACUUCGATT |
| as | GUUCUUCUGGAAGUCAAGCTT |
| as-ps(even#) | GpsUUpsCUpsUCpsUGpsGApsAGpsUCpsAApsGCpsTT |
| as-ps(19-21) | GUUCUUCUGGAAGUCAAGpsCpsTpsT |
| as-ps(2) | GpsUUCUUCUGGAAGUCAAGCTT |
| GL2 as | UCGAAGUAUUCCGCGUACGTT |

B

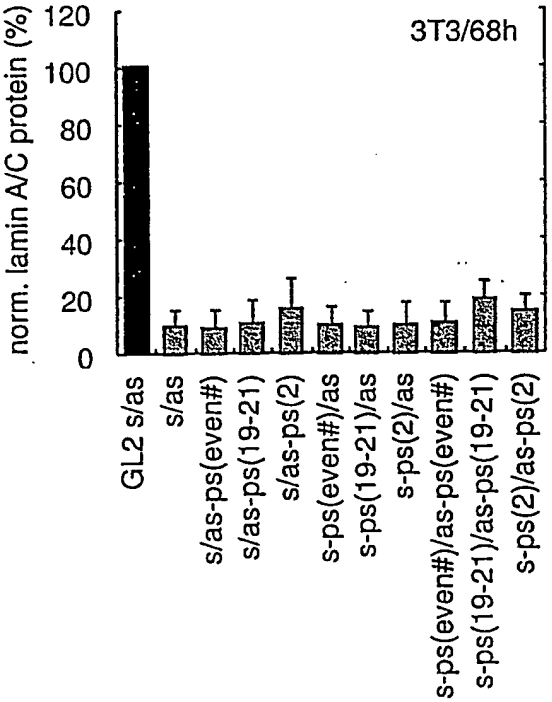


Figure 8 C,D

C

human target region (608-630)
AACUGGACUUCCAGAAGAACAUC

| | |
|---|---|
| s | CUGGACUUCCAGAAGAACATT |
| s-ps(19-21) | UCUGGACUUCCAGAAGAACpsApsTpsT |
| as | UGUUCUUCUGGAAGUCCAGTT |
| as-ps(19-21) | UGUUCUUCUGGAAGUCCApsGpsTpsT |

D

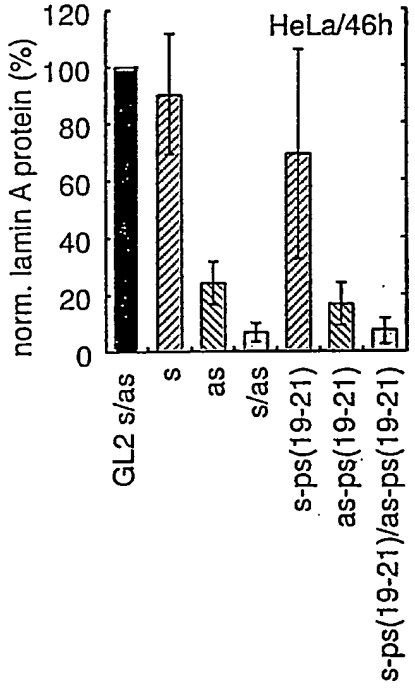


Figure 9

**A**

human target region (778-800)
AAGAAGGAGCUGGAGAAGACUUA

| | |
|---|---|
| s-5´-p | pGAAGGAGCUGGAGAAGACUTT |
| s-5´-p,ps | pGAAGGAGCUGGAGAAGACpsUpsTpsT |
| s-5´-p,f | pGAAGGAGfCfUGGAGAAGAfCfUTT |
| s-5´-p,f,ps | pGAAGGAGfCfUGGAGAAGAfCpsfUpsTpsT |
| as-5-p, | pAGUCUUCUCCAGCUCCUUCTT |
| as-5-p,ps | pAGUCUUCUCCAGCUCCUUpsCpsTpsT |
| as-5-p,f | pAGfUfCfUfUfCfUfCfCAGfCfUfCfCfUfUfCTT |
| as-5-p,f,ps | pAGfUfCfUfUfCfUfCfCAGfCfUfCfCfUfUpsfCpsTpsT |

**B**

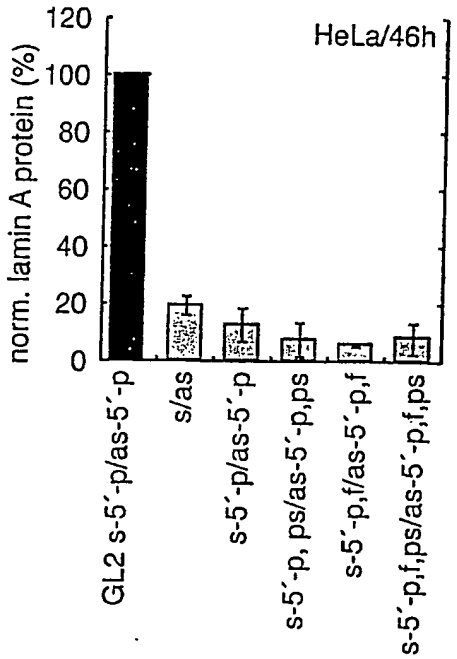
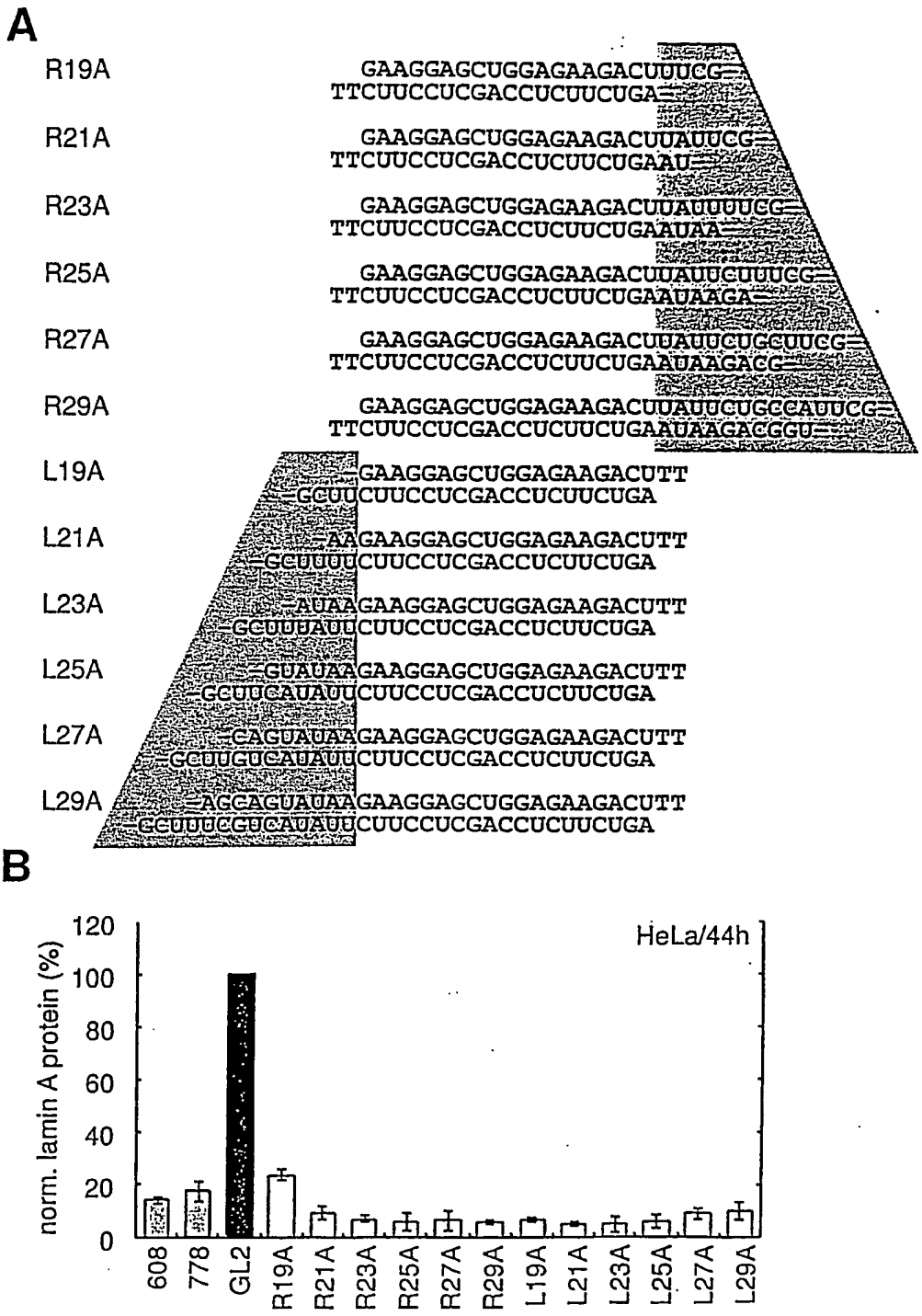


Figure 10


A
R19A
GAAGGAGCUGGAGAAGACUUUCG
TTCUUCCUCGACCUCUUCUGA
R21A
GAAGGAGCUGGAGAAGACUUAUUCG
TTCUUCCUCGACCUCUUCUGAAU
R23A
GAAGGAGCUGGAGAAGACUUAUUUUCG
TTCUUCCUCGACCUCUUCUGAAUAA
R25A
GAAGGAGCUGGAGAAGACUUAUUCUUUCG
TTCUUCCUCGACCUCUUCUGAAUAAGA
R27A
GAAGGAGCUGGAGAAGACUUAUUCUGCUUCG
TTCUUCCUCGACCUCUUCUGAAUAAGACG
R29A
GAAGGAGCUGGAGAAGACUUAUUCUGCCAUUCG
TTCUUCCUCGACCUCUUCUGAAUAAGACGGU
L19A
GAAGGAGCUGGAGAAGACUTT
GCUUCUUCCUCGACCUCUUCUGA
L21A
AAGAAGGAGCUGGAGAAGACUTT
GCUUUUCUUCCUCGACCUCUUCUGA
L23A
AUAAGAAGGAGCUGGAGAAGACUTT
GCUUUAUUCUUCCUCGACCUCUUCUGA
L25A
GUAUAAGAAGGAGCUGGAGAAGACUTT
GCUUCAUAUUCUUCCUCGACCUCUUCUGA
L27A
CAGUAUAAGAAGGAGCUGGAGAAGACUTT
GCUUGUCAUAUUCUUCCUCGACCUCUUCUGA
L29A
AGCAGUAUAAGAAGGAGCUGGAGAAGACUTT
GCUUUCGUCAUAUUCUUCCUCGACCUCUUCUGA
B
HeLa/44h
norm. lamin A protein (%)
120
100
80
60
40
20
0
608
778
GL2
R19A
R21A
R23A
R25A
R27A
R29A
L19A
L21A
L23A
L25A
L27A
L29A

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(54) Title: RNA INTERFERENCE BY PALINDROMIC OR MODIFIED RNA MOLECULES

(57) Abstract: The present invention relates to sequence and structural features of RNA molecules required to mediate target-specific nucleic acid modifications by RNA-interference (RNAi), such as target mRNA degradation and/or DNA methylation.

WO 2004/065600 A3

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/000314

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | MUJEEB A ET AL: "NMR structure of the mature dimer initiation complex of HIV-1 genomic RNA" FEBS LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 458, no. 3, 24 September 1999 (1999-09-24), pages 387-392, XP004260297 ISSN: 0014-5793 figure 1 ----- -/-- | 1,6,8, 10,12 |

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 15 June 2004 | 25. 10. 04 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Weikl, M |

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/000314

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | HAMADA M ET AL: "EFFECTS OF RNA INTERFERENCE IN GENE EXPRESSION (RNAI) IN CULTURED MAMMALAIN CELLS OF MISMATCHES AND THE INTRODUCTION OF CHEMICAL MODIFICATIONS AT THE 3'-ENDS OF SIRNAS" ANTISENSE & NUCLEIC ACID DRUG DEVELOPMENT, MARY ANN LIEBERT, INC., NEW YORK, US, vol. 12, no. 5, October 2002 (2002-10), pages 301-309, XP009006637 ISSN: 1087-2906 the whole document | 1-13, 30-38 |
| A | ELBASHIR S M ET AL: "Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate" EMBO JOURNAL, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 20, no. 23, 3 December 2001 (2001-12-03), pages 6877-6888, XP002225998 ISSN: 0261-4189 the whole document | 1-13, 30-38 |
| A | TUSCHL T: "Expanding small RNA interference" NATURE BIOTECHNOLOGY, NATURE PUBLISHING, US, vol. 20, no. 5, May 2002 (2002-05), pages 446-448, XP002232258 ISSN: 1087-0156 the whole document | 1-13, 30-38 |
| P,X | HARBORTH JENS ET AL: "Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing." ANTISENSE AND NUCLEIC ACID DRUG DEVELOPMENT, vol. 13, no. 2, April 2003 (2003-04), pages 83-105, XP002284355 ISSN: 1087-2906 (ISSN print) the whole document | 1-13, 30-38 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2004/000314

## Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-13 (completely) and 30-38 (partially)

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-13 (completely) and 30-38 (partially)

   Claims which relate to siRNAs with palindromic sequences

   ---

2. claims: 14-17 (completely) and 30-38 (partially)

   Claims which relate to siRNAs with labelling groups

   ---

3. claims: 18-22 (completely) and 30-38 (partially)

   Claims which relate to siRNAs with modifications which inhibit RNA interference

   ---

4. claims: 23-29 (completely) and 30-38 (partially)

   Claims which relate to siRNAs with one 2'-OH modified nucleotide and one modified phosphoester group

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